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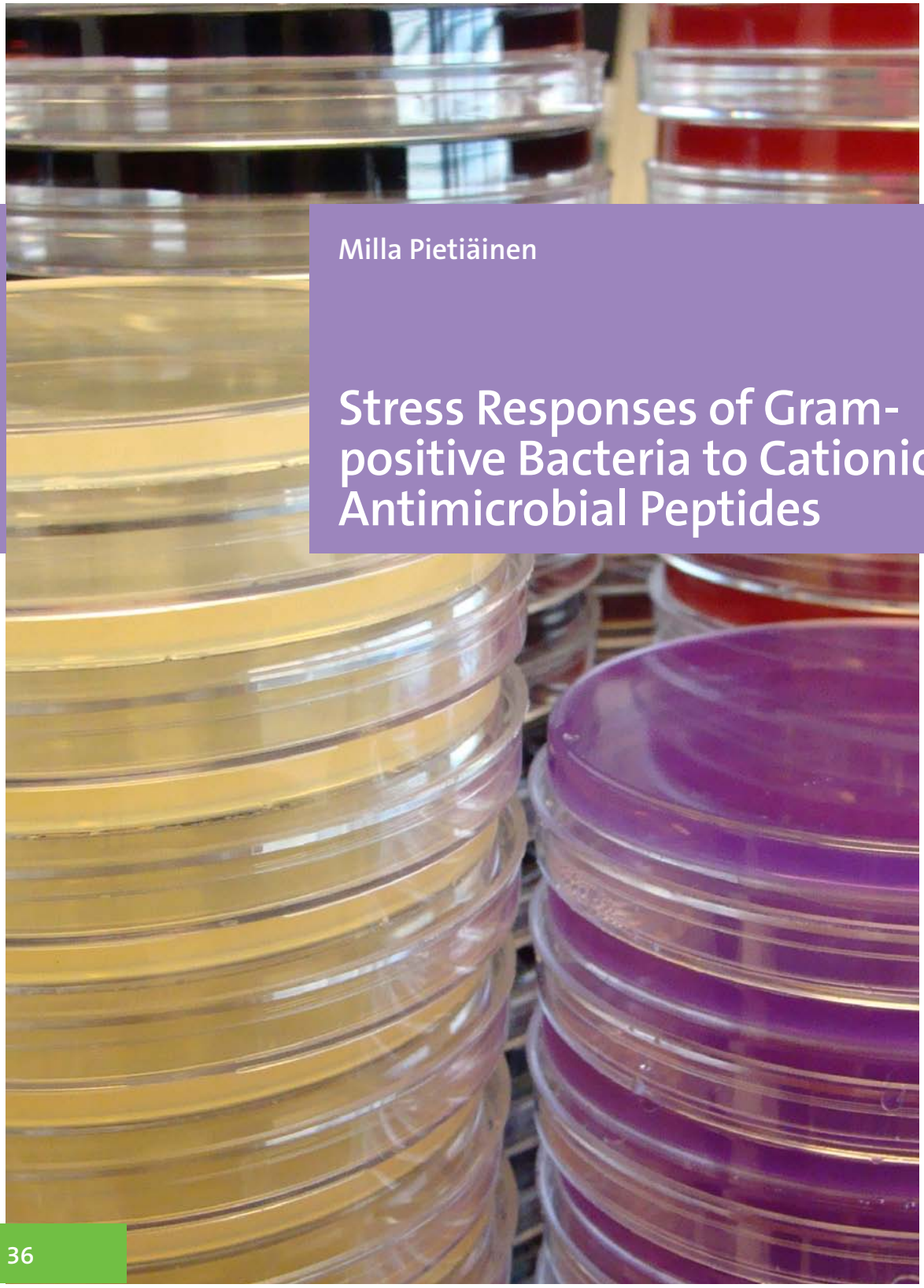

RESEARCH

Milla Pietiäinen

Stress Responses of Gram-positive Bacteria to Cationic Antimicrobial Peptides

RESEARCH 36

Milla Pietiäinen

Stress Responses of Gram-Positive Bacteria to Cationic Antimicrobial Peptides

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in Auditorium 1041, Biocenter 2, Viikinkaari 5, on September 17th, 2010, at 12 noon

National Institute for Health and Welfare, Helsinki, Finland
and
Faculty of Biological and Environmental Sciences, Department of Biosciences, Division of Genetics, University of Helsinki, Finland

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To my Parents

Abstract

Milla Pietiäinen. Stress Responses of Gram-Positive Bacteria to Cationic Antimicrobial Peptides. National Institute for Health and Welfare (THL), Research 36. 137 pages. Helsinki 2010.
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As the resistance of bacteria to conventional antibiotics has become an increasing problem, new antimicrobial drugs are urgently needed. One possible source of new antibacterial agents is a group of cationic antimicrobial peptides (CAMPs) produced by practically all living organisms. These peptides are typically small, amphipathic and positively charged and contain well defined α-helical or β-sheet secondary structures. The main antibacterial action mechanism of CAMPs is considered to be disruption of the cell membrane, but other targets of CAMPs also exist. Some bacterial species have evolved defence mechanisms against the harmful effects of CAMPs. One of the most effective defence mechanisms is reduction of the net negative charge of bacterial cell surfaces.

Global analysis of gene expression of two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, was used to further study the stress responses induced by different types of CAMPs. *B. subtilis* cells were treated with sublethal concentrations of α-helical peptide LL-37, β-sheet peptide protegrin 1 or synthetic analogue poly-L-lysine, and the changes in gene expression were studied using DNA macroarrays. In the case of *S. aureus*, three different α-helical peptides were selected for the transcriptome analyses: temporin L, ovispirin-1 and dermaseptin K4-S4(1-16). Transcriptional changes caused by peptide stress were examined using oligo DNA microarrays.

The transcriptome analysis revealed two main cell signalling mechanisms mediating CAMP stress responses in Gram-positive bacteria: extracytoplasmic function (ECF) sigma factors and two-component systems (TCSs). In *B. subtilis*, ECF sigma factors σ^W and σ^M as well as TCS LiaRS responded to the cell membrane disruption caused by CAMPs. In *S. aureus*, CAMPs caused a similar stress response to antibiotics interfering in cell wall synthesis, and TCS VraSR was strongly activated. All of these transcriptional regulators are known to respond to several compounds other than CAMPs interfering with cell envelope integrity, suggesting that they sense cell envelope stress in general.

Among the most strongly induced genes were *yxdLM* (in *B. subtilis*) and *vraDE* (in *S. aureus*) encoding homologous ABC transporters. Transcription of *yxdLM* and *vraDE* operons is controlled by TCSs YxdJK and ApsRS, respectively. These TCSs seemed to be responsible for the direct recognition of CAMPs. The *yxdLM* operon was specifically induced by LL-37, but its role in CAMP resistance remained unclear. VraDE was proven to be a bacitracin transporter.

We also showed that the net positive charge of the cell wall affects the signal recognition of different TCSs responding to cell envelope stress. Inactivation of the

Dlt system responsible for the D-alanylation of teichoic acids had a strong and differential effect on the activity of the studied TCSs, depending on their functional role in cells and the stimuli they sense.

Keywords: cationic antimicrobial peptide, stress response, two-component system, sigma factor, transcriptome analysis, *Bacillus subtilis*, *Staphylococcus aureus*

Tiivistelmä

Milla Pietiäinen. Stress Responses of Gram-Positive Bacteria to Cationic Antimicrobial Peptides [Kationisten antimikrobipeptidien aiheuttamat stressivasteet Gram-positiivisissa bakteereissa]. Terveyden ja hyvinvoinnin laitos (THL), Tutkimus 36. 137 sivua. Helsinki 2010.
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Bakteerien vastustuskyvystä perinteisiä antibiootteja vastaan on tulossa kasvava ongelma ja uusia antimikrobilääkkeitä tarvitaan pikaisesti. Kationiset antimikrobipeptidit (CAMP) ovat eräs mahdollinen vaihtoehto uusiksi lääkkeiksi. Näitä peptideitä tuottavat lähes kaikki elävät organismit ja niillä on useita tyypillisiä piirteitä kuten pieni koko, amfipaattisuus sekä positiviinen varaus. Lisäksi kyseiset peptidit sisältävät usein niille tyypillisiä α-helikaalisia tai β-levy sekundäärirakenteita. Kationiset peptidit tuhoavat bakteereita pääasiallisesti häiritsemällä niiden solukalvon rakennetta, mutta niillä on myös muita vaikutuskohteita. Jotkin bakteerilajit ovat pystyneet kehittämään puolustumekanismeja antimikrobipeptidejä vastaan. Eräs tärkeimmistä tällaisista mekanismeista on bakteerin pintarakenteiden positiivisen varauksen kasvattaminen.

Erilaisten kationisten antimikrobipeptidien aiheuttamaa stressivastetta ja muutosta geeniekspressiossa tutkittiin kahdessa Gram-positiivisessa mikrobissa, *Bacillus subtilis*- ja *Staphylococcus aureus*- bakteerissa. *B. subtilis* -bakteerisolut käsiteltiin joko LL-37:llä (α-helikaalinen peptidi), protegriini 1:llä (β-levy peptidi) tai synteettisellä analogilla, poly-L-lysiinillä ja niiden aiheuttamia muutoksia geenien ilmenemiseen tutkittiin DNA-makroarray-tekniikkalla. *S. aureus*-bakteerin kohdalla transkriptomianalyysiin valittiin kolme erilaista α-helikaalista peptidiä: temporin L, ovispirin-1 ja dermaseptin K4-S4(1-16). Peptidien aiheuttamia muutoksia transkriptiossa tutkittiin oligo-DNA-mikroarray tekniikkalla.

Transkriptomianalyysit paljastivat kaksi pääasiallista peptidistressiin reagoivaa solun signaalivälityssysteemiä, ECF-sigmafaktorit sekä kaksikomponenttisysteemit (TCS). *B. subtiliksessa* ECF-sigmafaktorit σ^W ja σ^M sekä kaksikomponentti-systeemi LiaRS reagoivat peptidien aiheuttamiin häiriöihin solumembraanissa. *S. aureus*-bakteerissa peptidit aiheuttivat hyvin samankaltaisen stressivasteen kuin soluseinän synteesiä häiritsevät antibiootit aiheuttaen kaksikomponenttisysteemi VraSR:n voimakkaan aktivoitumisen. Kaikki nämä transkription säätelijät reagoivat useisiin erilaisiin solun seinämää häiritseviin yhdisteisiin eli ne todennäköisesti aistivat yleisesti soluseinämän stressitilaa.

Voimakkaimmin indusoituneita geenejä olivat *yxdLM* (*B. subtilis*) ja *vraDE* (*S. aureus*), jotka koodaavat toistensa kaltaisia ABC-kuljettajaproteiineja. *yxdLM* ja *vraDE* operonien transkriptiota säätelee kaksikomponettisysteemit YxdJK ja ApsRS ja ne mitä ilmeisimmin reagoivat spesifisesti antimikrobipeptidien läsnäoloon. *yxdLM* aktivoitui spesifisesti LL-37:n vaikutuksesta, mutta sen rooli peptidi-

resistenssisä jäi epäselväksi. Tulokset osoittivat, että VraDE on basitrasiinin kuljettaja.

Osoitimme myös, että soluseinän positiivinen varaus vaikuttaa soluseinämän stressiä aistivien kaksikomponenttisysteemien signaalin tunnistukseen. Dlt-systeemi on vastuussa teikkohappojen D-alanylaatiosta ja tämän systeemin inaktivaatiolla oli suuri vaikutus tutkittavien kaksikomponenttisysteemien aktiivisuuteen. Vaikutus vaihteli riippuen siitä mikä kunkin kaksikomponenttisysteemin funktio on solussa ja minkälaisia aktivoivia signaaleja ne tunnistavat.

Avainsanat: kationinen antimikrobipeptidi, transkriptomianalyysi, stressivaste, kaksikomponenttisysteemi, sigmafaktori, *Bacillus subtilis*, *Staphylococcus aureus*

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Original publications

List of original papers

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I **Milla Pietiäinen**, Marika Gardemeister, Maria Mecklin, Soile Leskelä, Matti Sarvas and Vesa P. Kontinen (2005): Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems. Microbiology 151: 1577–1592

II Hanne-Leena Hyyryläinen, **Milla Pietiäinen**, Tuula Lunden, Anna Ekman, Marika Gardemeister, Sanna Murtomäki-Repo, Haike Antelmann, Michael Hecker, Leena Valmu, Matti Sarvas and Vesa P. Kontinen (2007): The density of negative charge in the cell wall influences two-component signal transduction in *Bacillus subtilis*. Microbiology 153: 2126–2136

III **Milla Pietiäinen**, Patrice François, Hanne-Leena Hyyryläinen, Manuela Tangomo, Vera Sass, Hans-Georg Sahl, Jacques Schrenzel and Vesa P. Kontinen (2009): Transcriptome analysis of the responses of *Staphylococcus aureus* to antimicrobial peptides and characterization of the roles of *vraDE* and *vraSR* in antimicrobial resistance. BMC Genomics 10:429

Abbreviations

aa	amino acid
ABC	ATP-binding cassette
AmyQ	α-amylase of *Bacillus amyloliquefaciens*
ATP	adenosine triphosphate
CAMP	cationic antimicrobial peptide
D-ala	D-alanyl ester
ECF σ factor	Extracytoplasmic function sigma factor
EGTA	ethylene glycol tetraacetic acid
GlcNAc	N-acetylglucosamine
HK	histidine kinase
IPTG	isopropyl-β-D-1-thiogalactopyranoside
LPG	lysylphosphatidylglycerol
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MATE	multidrug and toxic compound extrusion
MDR	multidrug resistance
MFS	major facilitator
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MurNAc	N-acetylmuramic acid
ORF	open reading frame
PBP	penicillin binding protein
PCR	polymerase chain reaction
PG	peptidoglycan
PG-1	protegrin 1
PLL	poly-L-lysine
PMF	proton motive force
qRT-PCR	quantitative real-time reverse transcription PCR
RND	resistance-nodulation-cell division
RR	response regulator
SMR	small MDR family
TA	teichoic acid
TCS	two-component system
VISA	vancomycin intermediate-resistant *S. aureus*
VRSA	vancomycin-resistant *S. aureus*
WTA	wall teichoic acid

.

1 Introduction

Cationic antimicrobial peptides (CAMPs) are produced by practically all living organisms, from the unicellular bacteria to the higher organisms in plant and animal kingdoms, and they act as an important part of innate immunity. The ever-growing problem of bacterial resistance to antibiotics used in health care has raised CAMPs as a subject of great scientific interest and it is hoped that they will provide potential new antimicrobial drugs. So far, over 1000 natural CAMPs have been identified and extensive research has also been performed to produce *de novo* designed peptides (Hale & Hancock, 2007).

Considering the long co-existence of pathogenic bacteria and CAMPs produced by host cells, CAMPs have retained their antimicrobial effectiveness remarkably well. However, some specific bacterial counter-measures against CAMPs have evolved as a result of dynamic co-evolutionary processes (Peschel & Sahl, 2006). One of the generally accepted killing mechanisms of CAMPs is that they attack the cell membrane of bacterial cells. The close monitoring and maintenance of cell envelope integrity is crucial for the survival of bacteria in changing environmental conditions, and especially in the presence of substances that interfere with the cell envelope (Jordan *et al.*, 2008).

In this thesis, two bacterial species belonging to the *Firmicutes* branch of Gram-positive bacteria were exposed to stress caused by CAMPs of human and animal origin, and the effects on gene expression were analyzed. Despite the phylogenetic relationship between the selected bacterial species, *Bacillus subtilis* and *Staphylococcus aureus*, they represent microbes living in completely different ecological niches. This gives an opportunity to identify the universal stress responses against CAMPs among Gram-positive bacteria, as well as determine the special features reflecting the demands of different habitats for the bacterial species. Understanding the bacterial mechanisms of resistance against compounds interfering with the cell envelope might help in identifying new targets for future antimicrobials.

B. subtilis is an apathogenic endospore-forming rod-shaped bacterium living in the soil. The majority of classical antibiotics are produced by microorganisms of the soil biosphere (Berdy, 2005), making soil an especially challenging habitat. The production of antimicrobial compounds is thought to give a competitive advantage against other microorganisms struggling for the limited resources. For example, more than two dozen antimicrobial agents with variable structures are produced by different *B. subtilis* species (Stein, 2005). Bacterial species of the genus *Bacillus* are also important industrial enzyme producers. Following the development of advanced molecular genetic tools, *B. subtilis* has become the best characterized species of the genus *Bacillus* and is often referred as the model Gram-positive bacterium in the field of molecular bacteriology.

S. aureus is a facultative anaerobic coccus belonging to the normal microbial flora of humans and animals. However, as an opportunistic pathogen, *S. aureus* is one of the most important bacteria causing diseases in humans. The anterior nares are the primary ecological niches of *S. aureus*, and nasal carriage has been identified as a major risk for infections, especially in clinical settings (Corbella *et al.*, 1997; Kluytmans *et al.*, 1995; von Eiff *et al.*, 2001). Resistance against several host-produced CAMPs is suggested to be a prerequisite for the ability of *S. aureus* to colonize the skin and other epithelial tissues (Foster, 2005; Peschel & Sahl, 2006). *S. aureus* can cause a variety of diseases, including skin and soft tissue infections, endocarditis, osteomyelitis, septic arthritis and pneumonia. In addition, *S. aureus* is often associated with catheter-related infections and postoperative wound infections. A wide range of virulence factors produced by *S. aureus* have been identified. These include both cell surface-associated proteins important in bacterial adhesion to host cells as well as secreted proteins such as proteases, lipases and several exo- and enterotoxins (Dinges *et al.*, 2000; Foster & Hook, 1998).

The emergence of antibiotic resistant strains of *S. aureus* has become an increasing problem. Practically all *S. aureus* strains isolated today produce β-lactamase and are therefore resistant to β-lactam antibiotics such as penicillin. A number of semisynthetic penicillins able to withstand bacterial β-lactamase, referred to as staphylococcal penicillins, were developed at the beginning of the 1960s. Soon after the first staphylococcal penicillin, methicillin, was introduced into clinical practice, the first methicillin-resistant *S. aureus* isolates (MRSA) were described (Jevons, 1961). Today, MRSA is the most commonly identified antibiotic-resistant pathogen worldwide. Methicillin resistance is provided by the mobile chromosomal cassette SCC*mec* carrying the *mecA* gene encoding an altered penicillin-binding protein called PBP2´or PBP2A (Hartman & Tomasz, 1984; Matsuhashi *et al.*, 1986; Ubukata *et al.*, 1985). Different types of SCC*mec* cassettes can also contain genes providing resistance against several antibiotics other than β-lactams. The glycopeptide antibiotic vancomycin has been the primary drug used in the treatment of MRSA infections. However, the first vancomycin-intermediate MRSA strain (VISA) with reduced susceptibility to vancomycin due to the thickened cell wall was isolated in 1996 (Hiramatsu *et al.*, 1997; Sieradzki & Tomasz, 2003). The feared transfer of the plasmid-encoded vancomycin resistance gene *vanA* from *Enterococcus feacalis* took place, as the first clinical vancomycin-resistant MRSA strain (VRSA) was isolated in 2002 in the United States (Chang *et al.*, 2003).

2 Review of the literature

2.1 Cationic antimicrobial peptides – Host defence peptides

The existence of antimicrobial compounds in blood, secretions and various tissues was discovered at the beginning of the last century. The isolated compounds included bacteriolytic substances such as lysozyme, basic antimicrobial proteins and basic linear polypeptides. Even though the identity of the peptides was unclear, the idea of the interaction of these peptides with negatively charged cell surfaces and the disruption of important cell functions was soon established (reviewed by Brogden, 2005). The first isolated and purified cationic antimicrobial peptides included insect cecropins (Steiner *et al.*, 1981), amphibian magainins (Zasloff, 1987) and mammalian defensins (Ganz *et al.*, 1990), and several hundreds of antimicrobial peptides have since been identified. For long time, membrane disruption was thought to be the only or at least the major mechanism of action of these peptides, but recent studies have clearly shown that many of these peptides have alternative ways of causing cell death, for example through internal targets (Brogden, 2005; Hale & Hancock, 2007; Yeaman & Yount, 2003; Yount *et al.*, 2006). Several further roles of these peptides in addition to killing microbes have been discovered, such as in the immune response, wound healing and angiogenesis (Mookherjee & Hancock, 2007).

It has been suggested that the term 'cationic antimicrobial peptide' should only be used in cases where microbial killing has been proven to be the main function of the particular peptide. Otherwise, the term 'host defence peptide' would be more accurate (Hale & Hancock, 2007). For clarity, the term cationic antimicrobial peptide (CAMP) is used throughout the text in this thesis.

2.1.1 Diversity of antimicrobial peptides

Cationic antimicrobial peptides play a crucial role in innate immunity in all biological kingdoms and are produced in many tissues and cell types. CAMPs have a very broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, protozoa as well as some enveloped viruses such as influenza A and human immunodeficiency virus (HIV-1) (Jenssen *et al.*, 2006). CAMPs are an extremely diverse group of peptides having very little homology in their primary sequences, but they share some common features important to their function. CAMPs are generally 12-50 amino acids long and despite their small size they are usually gene-encoded and expressed either constitutively or inducibly. All CAMPs are proteolytically spliced from larger precursors including a signal sequence for secretion, and may go through post-translational modifications such as glycosylation, carboxy-terminal amidation, halogenization or cyclization (Zasloff, 2002). Their amino acid sequences often contain the basic amino acids lysine or arginine, giving the net positive charge characteristic of antimicrobial peptides, and

a substantial proportion of the hydrophobic residues alanine, leucine, phenylalanine or tryptophan. CAMPs are usually divided into subgroups on the basis of their secondary structure. These structural groups include α-helical linear peptides, β-sheet peptides, peptides containing extended structures rich in certain amino acids and looped peptides (Hancock, 2001) (Figure 1). The α-helical and β-sheet molecules are by far the most common antimicrobial peptides in nature. In addition to these "classical" groups of small peptides, polypeptides or larger proteins have also been shown to have antimicrobial activity. In addition, bacterial cells can produce CAMPs, often referred as bacteriocins. It is notable that anionic antimicrobial peptides also exist, as exemplified by human dermcidin (Schittek *et al.*, 2001) and maximin H5 from amphibians (Lai *et al.*, 2002).

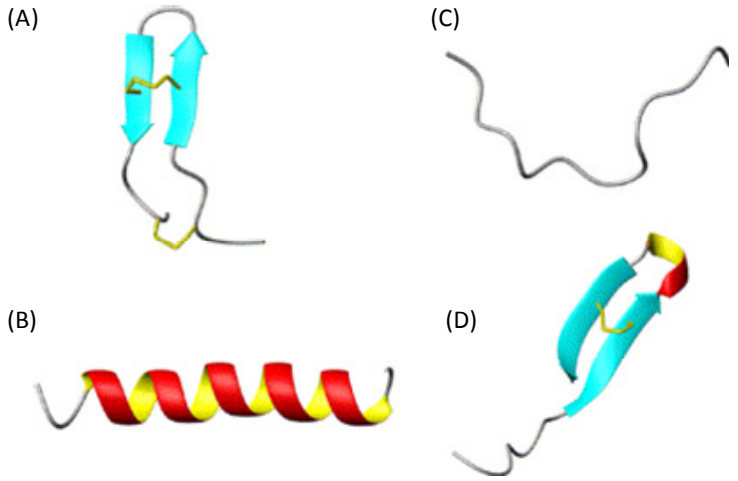

***Figure 1. Structural classes of CAMPs*:** (A) β-sheet peptides, tachyplesin I; (B) α-helical peptides, magainin 2; (C) peptides with extended structures, indolicidin; (D) looped peptides, thanatin. Adapted from Powers and Hancock (2003).

Linear α-helical peptides

Linear α-helical peptides comprise one of the two largest groups of antimicrobial peptides and include several hundreds of peptides present in both invertebrates and vertebrates (Bulet *et al.*, 2004). The skin of a variety of frog species is an especially rich source of α-helical peptides. For example, 50 and 76 peptides belonging to the dermaseptins and temporins, respectively, have been characterized to date (Mahalka & Kinnunen, 2009; Nicolas & El Amri, 2009). Typical for the peptides of both families is a highly variable antimicrobial region and a conserved preproregion of the precursors. Although β-sheet peptides are the most abundant peptides in mammals, several α-helical peptides belonging to cathelicidin family have also been found (Bals & Wilson, 2003; Bulet *et al.*, 2004; Gennaro & Zanetti, 2000; Zanetti,

2004). Cathelicidins are derived from prepropeptides, having a well-conserved N-terminal propeptide segment. Several different cathelicidins are usually produced by an individual mammalian species, the exception being humans, which express only one cathelicidin, LL-37. LL-37 has broad antimicrobial and immunomodulatory activity and is primarily produced by phagocytic leukocytes and epithelial cells (Durr *et al.*, 2006).

α-Helical peptides are usually 12-25 residues long and the α-helices often contain a slight bend in the center of the molecule that might have a role in suppressing haemolytic activity (Zhang *et al.*, 1999). Crucial for their function seems to be the lack of structure in aqueous solutions, but an inducible structure when interacting with the hydrophobic environment of membranes (Chen *et al.*, 2005b; Lee *et al.*, 2003). Another important factor is the ability to self-associate (i.e. the ability to oligomerize/dimerize). Several studies have shown that self-association of α-helical peptides in the membrane-bound state correlates with antimicrobial activity (Strahilevitz *et al.*, 1994), but self-association in the aqueous environment may in fact interfere with antimicrobial activity (Chen *et al.*, 2005b; Lee *et al.*, 2003).

β-sheet peptides

The second class of antimicrobial peptides consists of a highly diverse group of peptides at the level of primary structure. β-Sheet peptides contain cysteine residues and form more restrained conformations characterized by the presence of a variable number of antiparallel β-strands stabilised by a series of intramolecular disulphide bonds. Depending on the quantity of cysteine residues and the overall length of the peptide, they can adopt either a β-sheet conformation with triple strands, as in the case of most verterbrate defensins, or a β-hairpin structure seen, for example, in porcine protegrins. Larger peptides may also contain minor helical segments, as in invertebrate defensins and mammalian β-defensins (Bulet *et al.*, 2004). The number of disulphide bridges usually varies from one to four; for example, bactenecin from bovine neutrophils contains one, protegrin-1 from porcine leukocytes and tachyplesins from horseshoe crabs two, mammalian and insect defensins three and plant defensins up to four disulphide bonds (Bulet *et al.*, 2004; Carvalho Ade & Gomes, 2009; Ganz & Lehrer, 1995; Kokryakov *et al.*, 1993; Lehrer, 2004; Romeo *et al.*, 1988). The anti-fungal peptide mytimicin from the blue mussel contains as many as 12 cysteine residues forming intramolecular disulphide bridges (Charlet *et al.*, 1996). Recently, a common motif present in all cysteine-stabilized antimicrobial peptides was discovered. Characteristic of this γ-motif is two cysteine-stabilized antiparallel β-sheets and the distribution of basic residues in the poles of the motif (Yeaman & Yount, 2007; Yount & Yeaman, 2004).

Defensins are one of the most important groups of CAMPs in mammals. They are divided into three sub-groups, α-, β- and θ–defensins. The classification of α- and β- defensins is based on the peptide precursor and gene structures, as well as the placement and disulphide pairings of the six conserved cysteine residues. α-Defensins are mainly produced constitutively by neutrophils, macrophages and intestinal Paneth cells, whereas β-defensins are inducibly expressed in epithelial

tissues (Selsted & Ouellette, 2005). The structure of θ-defensins is exceptional among mammalian CAMPs, resulting from the head-to-tail cyclization of the peptide backbone. θ-Defensins are produced from mutated α-defensin genes (Leonova *et al.*, 2001; Tang *et al.*, 1999), and they are present in several species of Old World monkeys and orangutans, but not in humans (Nguyen *et al.*, 2003).

Like α-helical peptides, β-sheet peptides are also amphipathic, but in contrast to α-helical peptides they often exist as dimers in aqueous solutions. The mechanisms by which these kinds of peptides interact and cause membrane disruption are still poorly understood. It was assumed that intramolecular disulphide bridges are essential for the antimicrobial activitity, but it seems that their main function is to protect peptides from proteolysis (Selsted & Ouellette, 2005).

Extended peptides rich in certain amino acids

The class of peptides with extended structures contains peptides lacking classical secondary structures due to their unusual amino acid composition, rich in one or more specific amino acids. For example, in the case of human salivary histatins, the peptides are rich in histidin residues, whereas indolicin from bovine neutrophils has a high content of tryptophan and proline (Oppenheim *et al.*, 1988; Selsted *et al.*, 1992). The role of membrane disruption as a mechanism of action in the case of these peptides has not been completely elucidated. Model membrane studies with indolicidin have shown that it is not effectively translocated across membranes, and it was also unable to completely depolarize the cytoplasmic membrane of *E. coli* and *S. aureus* (Friedrich *et al.*, 2000; Wu *et al.*, 1999). Histatins seem to have intracellular targets (Helmerhorst *et al.*, 1999).

Looped peptides

In contrast to other antimicrobial peptides, looped peptides cannot form amphipathic structures due to their proline-arginine rich sequence. The looped structure is formed by a single disulphide, amide or isopeptide bond (Powers & Hancock, 2003). It is thought that members of this group might also have other targets than the cell membrane. For example, the looped peptide thanatin does not induce changes in membrane permeability and D- and L- enantiomers possess different kinds of antimicrobial activity, suggesting an involment of specific receptor molecules (Fehlbaum *et al.*, 1996). Lantibiotics produced by Gram-positive bacteria are also often considered to belong to the class of looped peptides. These peptides contain small ring structures enclosed by a thioether bond (see below).

Protein-derived peptides

In addition to the classical small antimicrobial peptides, considerably larger polypeptides and proteins have been demonstrated to have antimicrobial activity. These proteins are usually proteolytically cleaved into several smaller fragments, some having a similar composition and structure to CAMPs. For example, lactoferricin derived from iron-binding glycoprotein lactoferrin present in mammalian milk and other fluid secretions has been reported to have direct antimicrobial activity (Gifford *et al.*, 2005). Another example is buforin I from the Asian toad, which is produced proteolytically from the histone protein H2A

normally associated with the nucleosome (Kim *et al.*, 1996). Peptides derived from proteins of the complement cascade also appear to have antibacterial activity (Nordahl *et al.*, 2004).

Chemokines are important immunomodulatory signalling molecules, but there is clear evidence indicating that they can also have a direct antimicrobial function. More than 30 chemokines have been reported to have either direct antibacterial or antifungal activity. These chemokines, often referred to as kinocidins, contain three structurally and functionally different domains: an N-terminal unstructured region responsible for the chemotactic trait, a central domain constructed by a triple-stranded β-sheet and a C-terminal α-helical domain contributing to antimicrobial action (Yount *et al.*, 2006). A body of evidence suggests that the α-helical domain may be cleaved from the parent protein in order to form a functional microbicide (Bjorstad *et al.*, 2005). Moreover, kinocidins contain the γ-core motif present in variety of disulphide-stabilized antimicrobial peptides, suggesting a close evolutionary relationship between kinocidins and antimicrobial peptides (Yount *et al.*, 2006).

Bacteriocins

Bacteriocins are antimicrobial peptides or proteins mainly synthesised by Gram-positive bacteria. The fundamental difference between bacteriocins and conventional antibiotics is that bacteriocins are gene-encoded and ribosomally synthesised. Based on their primary structure, bacteriocins are classified into three groups: class I, class II and class III bacteriocins (reviewed by Hechard & Sahl, 2002).

The class I bacteriocins include peptides that undergo extensive post-translational modifications such as the dehydration of serines and threonines to didehydroalanine and didehydrobutyrine, respectively, and (methyl) lanthione ring formation. Due to the presence of lanthione rings, peptides belonging to class I bacteriocins are referred to as lantibiotics. Lantibiotics can be further divided into group A and group B lantibiotics. Group A lantibiotics are linear, amphipathic and positively charged peptides, whereas group B consists of globular peptides. Nisin is the best characterized group A lantibiotic (Fig. 2), but this group also includes other extensively studied peptides such as subtilin, epidermin, gallidermin, Pep5 and lacticin481 (reviewed by Hechard & Sahl, 2002; Lubelski *et al.*, 2008). Lantibiotics are effective against a wide range of bacterial species and it seems that the killing of bacterial cells is often achieved via specific receptors. For example, nisin interacts with lipid II, which is a precursor in bacterial cell wall synthesis (Breukink & de Kruijff, 1999; Breukink *et al.*, 1999).

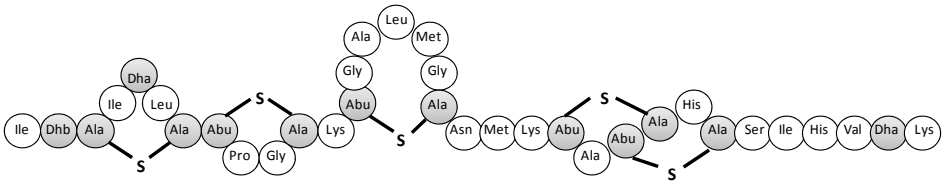

Figure 2. Structure of lantibiotic nisin. Dha = dehydroalanine, Dhb = dehydrobutyrine, Abu = aminobutyric acid, Ala-S-Ala = lanthionine, Abu-S-Ala = 3-methyllanthionine. Adapted from Hechard and Sahl (2002).

The class II bacteriocins are unmodified, cationic and hydrophobic peptides causing membrane permeabilisation in sensitive bacteria. In contrast to the class I bacteriocins, the class II bacteriocins are only effective against a narrow range of bacterial species that is limited to related species or strains of the producing bacteria. Pediocin PA-1 is one of the most extensively studied class II bacteriocins and it is postulated to cause the leakage of K+ cations from bacterial cells (Bhunia 1991). Class III includes large bacteriocins with poorly characterized mechanisms of action.

2.1.2 Antibacterial mechanism of action

The exact action mechanism of CAMPs has still not been established. For a long time, the sole mechanism was thought to be the interactions of CAMPs with the cytoplasmic membrane, causing increased membrane permeability and leading to the leakage of cytoplasmic components. However, increasing evidence suggests that alternative or additional targets also exist (Hale & Hancock, 2007; Yeaman & Yount, 2003). Studies supporting this theory have shown that some peptides can cause cell death without significant membrane perturbation. Based on this knowledge, it has even been proposed that CAMPs should be divided into two functional groups: membrane-disruptive and membrane-nondisruptive peptides (Powers & Hancock, 2003). This distinction is not completely clear, since peptides that attack membranes of some species may be membrane-nondisruptive in other species. Furthermore, many peptides have a multifunctional role, affecting both cell membranes as well as internal targets. However, whatever the actual target of peptides, the ability to interact with lipid bilayers is crucial for their action.

Structural determinants of antimicrobial activity

The most important features of CAMPs concerning their action against microbes are charge, amphipathicity and hydrophobicity. For simplicity, they are often considered as individual features, but *in vivo* they cannot be separated from each other.

As the target of CAMPs is the negatively charged bacterial membrane, it is not surprising that an increased positive net charge strongly correlates with the antimicrobial activity of CAMPs to a certain extent (Yeaman & Yount, 2003). On the other hand, cationicity also has a strong correlation with the toxic effects of CAMPs. For example, Ovispirin-1 is a peptide with a positive charge of +8 and is

highly toxic to mammalian cells (Sawai *et al.*, 2002). The majority of native CAMPs have a net charge ranging from +4 to +6, which may represent an optimal charge for activity (Tossi *et al.*, 2000).

Studies conducted with modified peptides have shown that within a certain range, the addition of positively charged residues can increase the efficacy of peptides (Tossi *et al.*, 2000). Studies with maganin 2 analogues demonstrated that an increase in the charge from +3 to +5 resulted in increased antibacterial activity against both Gram-positive and negative bacteria. A further increase in the charge to +6 and +7 caused a loss of antimicrobial activity and led to increased haemolytic activity (Dathe *et al.*, 2001). A similar pattern was observed in a study on the α-helical amphipathic peptide L-V13K (Chen *et al.*, 2007), in which a systematic increase in the charge from +4 to +8 made V13K analogues more active against bacteria, while having a low level of haemolytic activity. A further increase in the positive charge to +9 and +10 had a dramatic effect on the haemolytic activity. Contradictory results have also been presented, in which modulation of the net charge of cecropin/melittin analogues from +5 to +9 had no significant effect on antimicrobial activity (Scott *et al.*, 1999).

Nearly all CAMPs form amphipathic structures upon interaction with target membranes. In these structures, the positively charged residues are segregated on the hydrophilic face and the hydrophobic residues lie on the opposite face of the peptides. This kind of amphipathic structure can be achieved through different protein conformations. It is clear that both amphipathicity and hydrophobicity are necessary for the function of CAMPs. The positively charged polar face helps the peptide to reach the target membrane through electrostatic bonding between CAMPs and structures on the bacterial surface, and the nonpolar face of peptides then allows insertion into the lipid bilayer through hydrophobic interactions (Powers & Hancock, 2003; Yeaman & Yount, 2003).

Both amphipathicity and hydrophobicity influence peptide activity towards negatively charged membranes of bacterial cells, but they may have an even more profound effect on neutral membranes of eukaryotic cells. A high degree of amphipathicity is correlated with increased toxicity toward cells composed of neutral phospholipids (Dathe & Wieprecht, 1999). Peptide hydrophobicity is approximately 50% for most antimicrobial peptides. Hydrophobicity is required for effective membrane permeabilization, but increased hydrophobicity is also strongly correlated with eukaryotic cell toxicity and the loss of antimicrobial activity (Chen *et al.*, 2007; Yeaman & Yount, 2003).

Initial peptide interactions with the cell envelope

Whether the final target of a peptide is the cell membrane or some intracellular component, it has to find its way through the cell envelope. Some bacterial species may form a dense outermost layer under certain environmental conditions. This capsular layer or glycocalyx has an overall net negative charge due to its acidic constituents, including polysaccharides such as polyribitol phosphate or dextran sulphate (Yount *et al.*, 2006). It has been suggested that one function of capsular

polysaccharides may be the segregation and inactivation of CAMPs before they reach the cell membrane (Campos *et al.*, 2004; Yeaman & Yount, 2003).

It is generally accepted that peptides are first attracted to bacterial surfaces via electrostatic bonding. Gram-positive organisms are surrounded by a relatively thick peptidoglycan (PG) layer containing teichoic or teichuronic acids. In Gram-negative bacteria, the cell wall consists of a thin peptidoglycan layer surrounded by an outer membrane layer in which acidic lipopolysaccharide (LPS) predominates. In both cases, the anionic outer layer presents a strong attractant to negatively charged peptides. In fact, there is increasing evidence that the cell wall itself could be the primary target of some peptides, and the manner in which they interfere with the assembly or integrity of the cell wall may even resemble the mechanism of cell wall-active β-lactam antibiotics (Ginsburg, 2004; Yount *et al.*, 2006). The best characterized examples of peptides interfering with peptidoglycan assembly are the lantibiotics nisin and mersacidin, which directly target lipid II (Brotz *et al.*, 1998a; Brotz *et al.*, 1998b). Inhibition of peptidoglycan synthesis is also found in Gram-negative microbes. For example, seminial plasmin from bovine seminal plasma inhibits *E. coli* cell wall assembly (Chitnis & Prasad, 1990).

A strong correlation between the net positive charge of peptides and membrane binding activity has been demonstrated in several studies (Bessalle *et al.*, 1992; Dathe *et al.*, 2001; Matsuzaki *et al.*, 1997; Vaz Gomes *et al.*, 1993). These electrostatic forces act over relatively long distances, and it has been demonstrated that lysine and arginine form particularly strong electrostatic bonds with phosphate groups in the lipid bilayer (Mavri & Vogel, 1996). Cationic peptides are suggested to traverse through the outer cell membrane of Gram-negative bacteria by a mechanism termed "self-promoted uptake" (Hale & Hancock, 2007; Hancock, 1997). According to this model, the initial action of the peptide involves the competitive replacement of LPS-associated divalent cations stabilizing the outer membrane. This is due to the higher affinity of peptides for negatively charged LPS than native divalent cations such as Mg^{2+} and Ca^{2+} (Jenssen *et al.*, 2006). This causes instability in the outer membrane, allowing the translocation of peptides through the lipid bilayer.

Studies with D- and L-enantiomers of native and model peptides have shown that both enantiomers exhibit equivalent antimicrobial activities, leading to the conclusion that most peptides interact with membranes without any specific receptors being involved (Bland *et al.*, 2001; Chen *et al.*, 2006; Wade *et al.*, 1990). On the other hand, there are several important exceptions to this generalization. One of the best studied examples is the lantibiotic nisin. It recognizes and specifically binds to Lipid II, a membrane-anchored cell wall precursor essential for bacterial PG synthesis, and uses it as a "docking molecule" to form pores in membranes in a targeted manner with high efficiency (Breukink *et al.*, 1999; Brotz *et al.*, 1998b). In the absence of Lipid II, nisin can also bind to anionic lipids and insert itself between the phospholipid groups, but this is only observed in model systems with micromolar concentrations compared to the nanomolar concentrations needed *in vivo* (Breukink & de Kruijff, 2006). Some other specific peptide binding sites might also exist; for example, tachyplesin has been shown to have a specific affinity for

LPS (Hirakura *et al.*, 2002). Peptides with non-equivalent activities for native all-L peptides versus their all-D enantiomers have also been observed, suggesting receptor-type interactions (Fehlbaum *et al.*, 1996; Vunnam *et al.*, 1997).

Membrane-disruptive mechanisms of cationic antimicrobial peptides

The initial binding events between CAMPs and the cell membrane are driven by electrostatic forces, followed by conformational phase transition and the insertion of peptides into the membrane. The amphipathic nature of peptides facilitates insertion at the interface of the hydrophilic head groups and hydrophilic fatty acyl chains of the membrane phopholipids (Powers & Hancock, 2003; Yeaman & Yount, 2003). Membrane disruption only follows if a sufficient amount of peptide accumulates in the cell membrane, a phenomenon referred to as the "threshold concentration". The concentration needed for membrane dysfunction depends on several factors, including the biochemical properties of a particular peptide and the phospholipid composition, fluidity or size of the head groups (Lee *et al.*, 2005; Yang *et al.*, 2000). At low peptide-to-lipid ratios, inactive peptides are embedded parallel to the membrane surface, causing stretching of the membrane. After the critical peptide concentration is reached, peptides shift to the active state and are orientated perpendicular to the membrane, causing the pore formation (Chen *et al.*, 2003; Heller *et al.*, 1998; Ludtke *et al.*, 1994; Sharon *et al.*, 1999; Yang *et al.*, 2000).

Based on studies with artificial membranes, three separate permeabilization models have been proposed (Fig. 3). In the barrel-stave model, a variable number of helical peptide molecules are inserted into the membrane, forming a barrel-like bundle around a central pore (Ehrenstein & Lecar, 1977). The hydrophilic surfaces of aggregated peptides form the inner pore lining, while the hydrophobic peptide regions face towards the acyl chains of the membrane. Although the barrel-stave mechanim is the oldest model presented, only a few peptides, such as alamethicin, have been shown to cause pore formation in this manner (Beven *et al.*, 1999; Sansom, 1991; Zhang *et al.*, 2001).

In the toroidal pore model, inserted peptides cause bending of the lipid monolayers, leading to the formation of pores lined with both the inserted peptides and the phospholipid head groups (Matsuzaki *et al.*, 1996). This type of action has been proposed for magainin, protegrins and melittin (Hallock *et al.*, 2003; Matsuzaki *et al.*, 1996; Yang *et al.*, 2001). The main difference between the barrel-stave and toroidal pore mechanisms is that in the latter, CAMPs are always associated with the lipid head groups, also when forming pores.

The third commonly supported model is the carpet mechanism, in which peptides act like detergents, covering the membrane surface in a carpet-like manner and subsequently leading to membrane disruption without long-lasting channel formation (Pouny *et al.*, 1992). Numerous peptides, including cecropins, dermaseptins and ovispirin (Amiche *et al.*, 1999; Gazit *et al.*, 1995; Pouny *et al.*, 1992; Yamaguchi *et al.*, 2001), are predicted to act via the carpet mechanism.

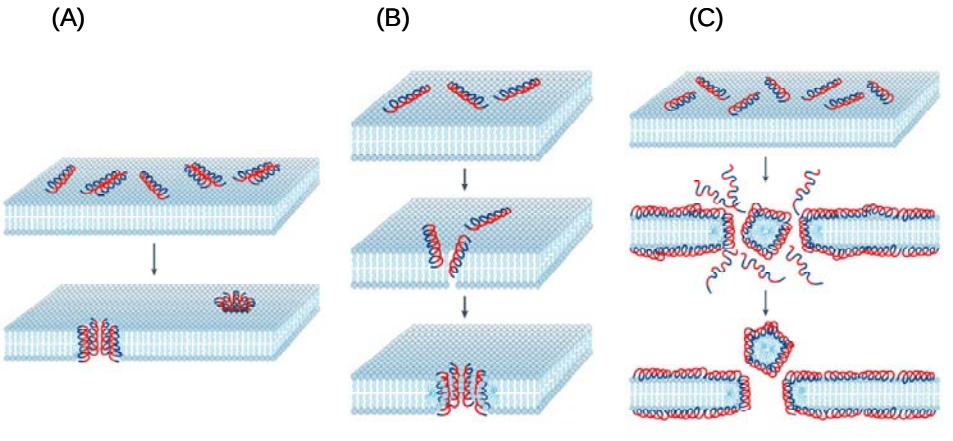

Figure 3. Models of membrane disruption mechanisms of CAMPs: (A) Barrel-stave mechanism; (B) Toroidal pore mechanism; (C) Carpet mechanism. Adapted from Brogden (2005).

It should be taken into consideration that none of the models presented above has been proven to be the actual mechanism acting *in vivo* (Brogden, 2005). It is possible that the actual membrane permeabilization of CAMPs involves elements from each of the models, and that the different models are in fact different stages of the same membrane rupture event (Dathe & Wieprecht, 1999; Papo & Shai, 2003). Recently, a new model termed "leaky slit" has also been presented, in which oligomerized peptides are suggested to form amphipathic membrane-spanning ribbons (Mahalka & Kinnunen, 2009; Zhao *et al.*, 2006). In this model, the hydrophobic side of the peptide ribbon faces towards the hydrocarbon chains of the lipid bilayer, while the hydrophilic side causes the formation of a positive curvature. This kind of action would be similar to that of amyloid-forming peptides and proteins in general. Cationic peptides predicted to act in this manner include temporin B and L, plantaricin A, magainin-2, sakacin P and dermaseptin S9 (Auvynet *et al.*, 2008; Zhao *et al.*, 2005; Zhao *et al.*, 2006)

Interestingly, the mode of membrane permeabilization might differ between mammalian and bacterial cells. For example, magainin forms small pores in the cell membrane of *Bacillus megaterium*, but causes more dramatic membrane disruption in ovary cells of the Chinese hamster (Imura *et al.*, 2008). Temporins 1Ta and Tl act in the barrel-stave manner when causing haemolysis, whereas their antimicrobial activity is achieved by the carpet mechanism (Carotenuto *et al.*, 2008).

Membrane-nondisruptive mechanisms of antimicrobial peptides

The clear dissociation between membrane permeabilization and cell death shown in several studies implies that alternative mechanisms to membrane dysfunction exist (Brogden, 2005; Hale & Hancock, 2007). CAMPs have even been suggested to have a "multitarget" mechanism of action (Powers & Hancock, 2003), describing their ability to interact with multiple anionic targets such as nucleic acids, cellular enzymes or constituents of the cell wall and membrane.

Due to the polyanionic nature of nucleic acids, it is not surprising that most CAMPs can bind to DNA and RNA with great avidity *in vitro*. One of the best characterized examples of CAMPs having microbicidal activity via DNA binding is buforin II, first isolated from the Asian toad (Park *et al.*, 1996). Buforin II is an α-helical linear peptide derived proteolytically from histone H2A and it has an ability to penetrate the cell membrane and kill bacterial cells by binding to DNA and RNA (Kim *et al.*, 2000; Park *et al.*, 1998a). Other histones reported to have antimicrobial activity are hipposin from shrimps (Birkemo *et al.*, 2003) and parasin I from catfish (Park *et al.*, 1998b). Another extensively studied example of DNA-binding CAMP is indolicin, a member of the cathelicidin family found in bovine neutrophils. For a long time it was thought to act through membrane permeabilization without cell lysis (Falla *et al.*, 1996), until its DNA-binding ability was discovered (Hsu *et al.*, 2005).

Many CAMPs can interfere in protein synthesis, folding or function. Direct inhibition of protein synthesis has been reported in studies on the pig cathelicidin PR39 and *E. coli* (Boman *et al.*, 1993), and a pleurocidin derivative from winter salmon restraining histidine incorporation in *E. coli* (Patrzykat *et al.*, 2002). Other peptides affecting protein synthesis include the α-helical peptides dermaseptin and pleurocidin and the human defensin HPN-1 (Jenssen *et al.*, 2006). Several studies have suggested that CAMPs cause the arrest of certain essential biosynthetic processes by binding and inactivating intracellular proteins. For example, correct folding of many proteins in *E. coli* is inhibited by the insect antimicrobial peptides pyrrhocoricin, drosocin and apidaecin, as the peptides bind to the bacterial chaperone DnaK (Kragol *et al.*, 2001; Otvos *et al.*, 2000). Another peptide, microsin B17, interferes in DNA replication by inactivating DNA gyrase of *E. coli* (del Castillo *et al.*, 2001).

Not only intracellular but also extracytoplasmic proteins such as autolysins can be targets for certain CAMPs. For instance, the lantibiotics nisin and Pep5 activates otherwise inert autolysins in cell wall extracts of staphylococci, which may be the explanation for cell lysis after peptide treatment (Bierbaum & Sahl, 1985; Bierbaum & Sahl, 1987). Inhibition of septum formation by CAMPs has also been postulated in studies with the lantibiotic microsin 25 interacting with membrane-associated septum-forming molecules in *E. coli* (Salomon & Farias, 1992). Inhibition of septum formation leads to filamentous cell formation, which is also seen in *E. coli* cells treated with indolicidin (Subbalakshmi & Sitaram, 1998) and *Salmonella typhimurium* cells treated with PR-39 and its truncated form PR-26 (Shi *et al.*, 1994).

Synergistic activity of CAMPs

Virtually all higher organisms produce more than one CAMP at a particular site, leading to the assumption that peptides may act co-operatively to kill bacteria. The enhanced antimicrobial activity of combinations of peptides compared to single peptides was first discovered in the dermaseptin family (Mor *et al.*, 1994). Recent studies have implied that the production of numerous structurally similar peptides within the same organism is a way to increase the antimicrobial spectrum by combining different peptide isoforms (Mangoni & Shai, 2009). For example, Gram-negative bacteria are resistant to the temporins 1Ta and Tb, but when either of them is combined with temporin 1Tl at sub-inhibitory concentrations, resistance is overcome (Rosenfeld *et al.*, 2006). Two out of three chicken gallinacins have also been demonstrated to act in synergy against *Salmonella enteriditis* (Milona *et al.*, 2007). More distinct peptides can also work together. For example, pig protegrin 1 has been shown to work in synergy with indolicidin, bactenesin or LL-37 (Yan & Hancock, 2001). Furthermore, human α- and β-defensins have been reported to act synergistically with LL-37 to kill *S. aureus* and *E. coli* cells (Chen *et al.*, 2005a; Nagaoka *et al.*, 2000).

CAMPs can assist in the uptake or enhance the antimicrobial effects of other microbicidal compounds such as traditional antibiotics or other host-derived antimicrobial proteins. For example, tachyplesin III combined with β-lactams or colistins inhibits the growth of multidrug-resistant *Pseudomonas aeruginosa* (Cirioni *et al.*, 2007). Synergistic interactions between CAMPs and enzymes are observed in some cases. Host-derived secretory phopholipase A of lacrimal fluid is an enzyme catalyzing the hydrolysis of the sn-2-ester-bond in phospholipids. In addition to this enzymatic function, it has antimicrobial activity of its own, which is strongly enhanced in the presence of magainin 2, indolicidin and temporins L and B (Zhao & Kinnunen, 2003).

Selective toxicity of CAMPs

It is widely accepted that at least some CAMPs exhibit selective toxicity, meaning that they can distinguish their target microbial cells from the host cells. The main features defining this selectivity are the composition of the cell membrane and membrane potential of the target cell (Yeaman & Yount, 2003). Bacterial cell membranes are rich in acidic phospholipids such as phosphatidylglycerol, cardiolipin and phosphatidylserine, giving a strong net negative charge to bacterial membranes. On the contrary, the outer leaflet of the plasma membrane in mammalian cells is mainly composed of zwitterionic phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. Due to these differences, CAMPs with a positive charge are more prone to binding to bacterial cell membranes. However, to a lesser extent there are also some negatively charged molecules on mammalian cell surfaces, such as gangliosides, which have been shown to have an essential role in the cellular entry of buforin IIb (Lee *et al.*, 2008). Not only the charge but also the composition of the cell membrane affects selectivity. For example, cholesterol is only present in mammalian membranes and stabilizes and protects the plasma membrane from attack by CAMPs. Another significant

distinction between prokaryotic and eukaryotic cells is the differences in their membrane potential. This electrochemical gradient results from proton flux across the membrane, and in normal mammalian cells it ranges from -90 to -110 mV, while in the case of bacterial cells it varies from -130 to -150 mV (Yeaman & Yount, 2003). The strong electronegative membrane potential may draw cationic peptides deeper into the microbial cell.

Recently, the issue of cell selectivity has been challenged and it has been suggested that all CAMPs should be considered as potentially toxic to mammalian cells in the absence of microbial targets (Matsuzaki, 2009). There is some clear evidence that CAMPs prefer microbial cells, as was shown in an experiment where dye-labelled magainin selectively bound to *S. aureus* but not to epithelial cells (Zasloff, unpublished work in Matsuzaki, 2009). It might be that CAMPs are less harmful to host cells simply by restricting their production in relatively inert host tissues such as epithelial cells, or inside the phogolysosomes of leukocytes (Matsuzaki, 2009; Yeaman & Yount, 2003).

2.1.3 Other roles of antimicrobial peptides

Many mammalian CAMPs lose their ability to kill bacterial cells under physiological conditions. It is known that antimicrobial activity is often antagonized by divalent and monovalent cations, glycosaminoglycans or mucins. However, CAMPs have been shown to cause an antimicrobial response *in vivo*, indicating that they may function as modulators of innate immunity. CAMPs have been reported to participate in the inactivation of endotoxin (LPS), they induce the production of several cytokines and chemokines and can also serve as chemokines (Mookherjee *et al.*, 2006a; Mookherjee & Hancock, 2007; Mookherjee *et al.*, 2007). They also play a role in cellular differentiation and proliferation, the suppression of apoptosis, wound repair and the stimulation of angiogenesis (Mookherjee & Hancock, 2007; Steinstraesser *et al.*, 2008). Furthermore, CAMPs can directly affect the regulation and expression of genes related to innate immunity (Mookherjee *et al.*, 2006b). A body of evidence suggests that CAMPs operate at the interface of innate and adaptive responses, serving as signals influencing the initiation, polarisation and amplification of adaptive immune responses (Bowdish *et al.*, 2005; Mookherjee & Hancock, 2007; Oppenheim *et al.*, 2003). A new interesting area of peptide research concerns the anticancer activities of CAMPs (Hoskin & Ramamoorthy, 2008).

2.1.4 Pharmaseutical use of antimicrobial peptides

As bacterial resistance to conventional antibiotics is an ever-growing problem, there is an urgent need to find or develop new antimicrobial drugs. Despite the great expectations of CAMPs being a potential new source of antibiotics, few CAMPs have been tested in clinical trials and their use has been restricted to topical applications (Hancock & Sahl, 2006; Oyston *et al.*, 2009). Four cationic antimicrobial peptides have been tested in phase 3 clinical efficacy trials. These include the magainin derivative Pexiganan for treating foot ulcers, a pig protegrin derivative Iseganan for oral mucosaitis, the human bactericidal permeability protein derivative Neuprex for sepsis and the indolicidin variant Omiganan for the treatment

of catheter-associated infections (Hancock & Sahl, 2006). However, only Omiganan has been approved for clinical use and is now pending licensing (Oyston *et al.*, 2009).

In addition to adequate efficacy, several problems concerning the drug usage of CAMPs exist. Possible toxicity to host cells, lability to proteases and high costs of production are factors limiting the clinical use of CAMPs. To overcome these challenges, considerable resources have been targeted at designing novel peptides with improved qualities. One potential source of new microbicidal compounds could be the antimicrobial peptides produced by Gram-positive bacteria. The nonribosomally synthesized peptides polymyxin B and gramicidin S have been in medical use for a long time and the lantibiotic nisin is used as a food additive to restrict bacterial growth. Nisin and lacticin 3147 are also used in veterinary medicine to prevent bovine mastitis (Ryan *et al.*, 1998). New candidates for medical use include the lantibiotic mutacin 1140 (Smith & Hillman, 2008).

2.2 Bacterial mechanisms for resisting antimicrobial peptides

Considering the ancient origin of CAMPs, surprisingly few bacterial species have developed highly effective resistance mechanisms against CAMPs. However, it seems that the co-evolution of CAMPs and bacterial resistance mechanisms has led to the emergence of a diverse repertoire of CAMPs (Peschel & Sahl, 2006; Zasloff, 2002). It has also been shown that resistance against a particular CAMP emerges through continued selection under laboratory conditions, although resistance is clearly more difficult to obtain than in the case of conventional antibiotics (Perron *et al.*, 2006). Certain bacterial species have exceptionally broad resistance to different kinds of CAMPs, including species belonging to genera such as *Serratia*, *Burkholderia*, *Morganella* and *Proteus* (Yeaman & Yount, 2003; Zasloff, 2002). These bacteria are protected against CAMPs by either stable structural or functional properties. Several important pathogens such as *Staphylococcus aureus* and *Salmonella enterica* have also evolved several strategies to avoid the toxicity of CAMPs. The most common bacterial resistance mechanisms include modifications of the cell envelope, the inactivation of CAMPs and active extrusion of harmful peptides.

2.2.1 Modifications of the cell envelope

Reduction of the net negative charge of the cell envelope

The initial interactions between bacteria and CAMPs are driven by electrostatic forces. From this point of view, it is not surprising that many of the resistance mechanisms used by bacteria are based on the reduction of the negative charge of the cell envelope. The essential molecules of the cell envelope can hardly be replaced without serious defects, but some bacteria are able to modify the charge of these molecules in order to prevent the attachment of CAMPs to the cell surface. Many Gram-positive bacteria such as *S. aureus*, *Streptococcus pyogenes*,

Streptococcus agalactiae and *Listeria monocytogens* are able to partially neutralize the negative charge of the cell wall by modifying teichoic acids with D-alanine (Abachin *et al.*, 2002; Kristian *et al.*, 2005; Peschel *et al.*, 1999; Poyart *et al.*, 2003). D-alanylation of teichoic acids is carried out by proteins encoded by the *dltABCD* operon found in many genomes of Gram-positive bacteria (Neuhaus & Baddiley, 2003; Perego *et al.*, 1995; Peschel *et al.*, 1999). It has been shown that *dlt*-deficient mutants are more susceptible to a broad variety of CAMPs and other cationic host defence factors (Koprivnjak *et al.*, 2002; Koprivnjak *et al.*, 2008; Kristian *et al.*, 2005; Peschel *et al.*, 1999). *S. aureus* also exploits another strategy to modify the charge of the cell envelope. The MprF catalyses the addition of L-lysine residues to the major membrane lipid phosphatidylglycerol, leading to the formation of positively charged lysyl-phosphatidylglycerol (LPG). Mutation of the *mprF* gene causes the same kind of phenotype as inactivation of the *dlt* operon (Peschel *et al.*, 2001). However, there appear to be certain limits after which modulation of the cell envelope charge is not sufficient to maintan resistance against CAMPs. Resistance may be overcome at high concentrations of CAMPs or by a high positive charge of a certain peptide, as in the case of human β-defensin hbD3 with a net positive charge of +10 (Harder *et al.*, 2001; Midorikawa *et al.*, 2003; Weidenmaier *et al.*, 2004). Reduction of the cell envelope charge is also a common mechanism in Gram-negative bacteria and is mainly achieved by modifications of lipid A in the outer membrane. The best characterised example is the incorporation of aminoarabinose into lipid A of *Salmonella enterica* and *Pseudomonas aeruginosa* (Ernst *et al.*, 2001; Gunn *et al.*, 1998).

Alterations in membrane structure and fluidity and reduction of the membrane potential

The susceptibility of bacteria to CAMPs is also influenced by physico-chemical properties of the cell membrane other than charge. It has been shown that the cell membranes of *S. aureus* strains resistant to tPMP-1 (a human kinocidin derived from platelets) contain elevated levels of longer-chain and unsaturated membrane lipids compared to tPMP-1-susceptible strains. This leads to higher membrane fluidity in resistant strains (Bayer *et al.*, 2000). It is postulated that the increased fluidity causes proton leakage out of the cells and consequently leads to a reduced membrane potential, interfering with the permeabilization of the peptides (Bayer *et al.*, 2006). On the other hand, reduced membrane fluidity due to an increased cardiolipin and lysyl-phosphatidylglycerol content in the membranes of *S. aureus* protoplasts is reported to associate with a reduced susceptibility to HNP-1, gramicidin D and tPMP-1 (Xiong *et al.*, 2005). It has been shown that in addition to the membrane stabilizing effect, cardiolipin also functions as a proton reservoir affecting the membrane potential (Kates *et al.*, 1993). The importance of membrane potential for the action of CAMPs has also been demonstrated with so-called small colony variants of *S. aureus* having mutations in the respiratory chain that cause a loss of membrane potential. A reduced membrane potential renders these *S. aureus* mutants more resistant to CAMPs (Yeaman & Bayer, 2006). The overall phospholipid composition and asymmetry between the two lipid layers in the bacterial cell membrane influences the susceptibility towards CAMPs. The outer leaflet of *S.*

aureus strains resistant to cationic molecules such as poly-L-lysine, cytocrome C and tPMP-1 contains significantly higher amounts of positively charged phospholipids compared to susceptible strains (Mukhopadhyay *et al.*, 2007).

2.2.2 Inactivation of CAMPs

α-Helical peptides are especially susceptible to proteolysis caused by bacterial peptidases and proteases. For example, the metalloprotease aureolysin and the serine protease V8 secreted by *S. aureus* are able to cleave LL-37. It has been shown that the production of these proteases is correlated with CAMP resistance *in vitro* (Sieprawska-Lupa *et al.*, 2004). In addition, the protease SepA of *S. epidermidis* has a similar function (Lai *et al.*, 2007). Many other species such as *Streptococcus pyogenes*, *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus mirabilis*, *Porphyromonas gingivalis* and *Prevotella* spp. produce CAMP-degrading proteases (Guina *et al.*, 2000; Nyberg *et al.*, 2004; Schmidtchen *et al.*, 2002).

CAMPs containing disulphide bridges or bacteriocins containing lanthionine amino acids are more resistant to proteolytic degradation. The presence of proline residues and amidation of the C-terminus also protects CAMPs from proteolysis. However, several bacterial species produce membrane bound metalloproteases involved in bacteriocin self-immunity, referred as Abi proteins (Kjos *et al.*, 2010). Non-nisin-producing *Lactococcus lactis* strains produce NSR protein which inactivates nisin by removing six amino acids from its carboxyl end (Sun *et al.*, 2009). Additional strategies to inactivate more stable CAMPs have also evolved among bacterial species. In contrast to peptidases and proteases, these mechanisms are usually specific for certain peptides. For example, the exoprotein staphylokinase produced by *S. aureus* can bind and inactivate α-defensins. In addition to its function in fibrinolysis, it also contributes to resistance to bacterial killing by α-defensins, and virulence (Jin *et al.*, 2004). *Streptococcus pyogenes* secretes SIC protein (**s**treptococcal **i**nhibitors of **c**omplement) and cell wall-bound M1 protein capable of binding LL-37 and other CAMPs with high affinity (Frick *et al.*, 2003; Peschel & Sahl, 2006). NisI immunity protein of *L. lactis* confers resistance to nisin and functions probably by intercepting nisin on the cell membrane (Qiao *et al.*, 1995; Stein *et al.*, 2003).

2.2.3 Extrusion of CAMPs

A general strategy of bacterial cells to avoid drugs or other toxic agents is their active extrusion from the cell or the cytoplasmic membrane. This active drug efflux is conducted against a concentration gradient via integral membrane proteins utilizing metabolic energy. Most efflux systems are specific for a narrow range of structurally related substrates, but systems exporting a broad spectrum of structurally dissimilar compounds also exist. These systems are referred to as multidrug resistance (MDR) transporters, and they are classified into two main groups based on the source of energy utilized in the translocation of toxic agents (Lubelski *et al.*, 2007). Primary transporters belong to the ATP binding cassette (ABC) superfamily hydrolysing ATP to provide free energy. Secondary exporters are drug/H+ or drug/Na+ antiporters utilizing proton motive force (PMF). Secondary

transporters can be further classified into several families according to their amino acid homology and secondary structure. These subclasses are the major facilitator superfamily (MFS), the small MDR family (SMR), the resistance-nodulation-cell division family (RND) and the multidrug and toxic compound extrusion family (MATE) (Fig. 4).

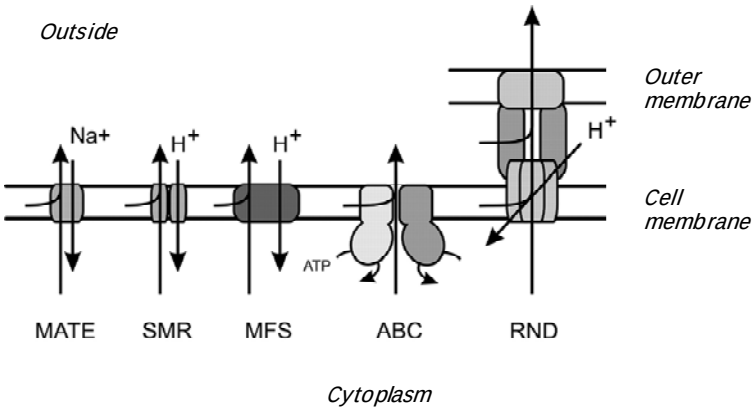

Figure 4. Major families of MDR transporters. Adapted from Lubelski (2007)

There is increasing evidence that secondary transporters are also important factors in bacterial resistance against CAMPs. One of the best characterised examples is the RND multidrug resistance transporter MtrCDE in *Neisseria gonorrhoeae* and *Neisseria meningitides*, conferring resistance to LL-37 and protegrins and various cell envelope stress-causing agents such as antibiotics, organic dyes and detergents (Shafer *et al.*, 1998; Tzeng *et al.*, 2005). In *Yersinia enterocolitica*, MFS transporter RosAB is also involved in CAMP resistance and it is believed to affect bacterial survival in neutrophils (Bengoechea & Skurnik, 2000). In *S. aureus*, the plasmid-encoded MFS exporter QacA is associated with resistance to tPMP-1, a human kinocidin derived from platelets (Kupferwasser *et al.*, 1999). However, it seems that this resistance phenotype is not caused by the efflux itself but rather results from the changes in the membrane fluidity and membrane potential caused by QacA (Bayer *et al.*, 2006).

Primary transporters of antimicrobial peptides have mainly been reported among Gram-positive bacteria producing bacteriocins themselves. For instance, *S. epidermidis* strains producing the lantibiotic epidermidin have evolved an additional protective system removing harmful peptides from the membrane after they have reached the lipid bilayer. This epidermidin resistance is mediated by the EpiEFG ABC transporter, which specifically binds only epidermidin and its very close relative, gallidermin (Otto *et al.*, 1998; Peschel & Gotz, 1996). Similar systems have been identified to export nisin from *L. lactis* and subtilin and mersacidin from *B. subtilis* (Altena *et al.*, 2000; Klein & Entian, 1994; Siegers & Entian, 1995).

2.3 How do Gram-positive bacteria sense cell envelope stress?

The cell envelope is an indispensable barrier protecting bacterial cells from environmental threats. The cell envelope gives cells their shape, withstands the high internal turgor pressure and mediates the controlled trafficking of proteins, ions and nutrients. It is also the target for numerous antibiotics, antimicrobial peptides and other antimicrobial compounds. For the survival of bacteria it is crucial that they can sense and rapidly react to environmental changes threathening cell envelope assembly, maintenance or function. In Gram-positive bacteria, two main cell signal transduction systems contribute to the sensing of cell envelope stress: two-component systems (TCSs) and alternative sigma (σ) factors (Jordan *et al.*, 2008).

2.3.1 Cell envelope of Gram-positive bacteria

The fundamental difference between Gram-positive and Gram-negative bacteria is the structure of their cell envelope. The cell envelope of Gram-negative bacteria consists of two lipid bilayers and a thin peptidoglycan sacculus between the membranes. In contrast, Gram-positive bacteria lack the outer membrane but have a substantially thicker cell wall with multiple peptidoglycan layers. In addition, many Gram-positive bacteria have other protective envelope structures such as extracellular polysaccharide capsules, S-layer proteins and mycolic acids (Weidenmaier & Peschel, 2008). Compared to Gram-negative bacteria, the cell envelope of Gram-positive bacteria comprises only two functional layers: a cell wall and cell membrane. Therefore, Gram-positive bacteria are generally considered to lack the periplasmic space present in the Gram-negative cell envelope between the peptidoglycan and inner membrane. However, this idea has recently been challenged by electron microscopy studies also showing the presence of a periplasmic-like space between the membrane and cell wall in Gram-postive bacteria (Matias & Beveridge, 2005; Matias & Beveridge, 2006). Either way, the cell wall-membrane interface of Gram-positive bacteria is the cell compartment in which several cellular processes take place.

Cell wall

The cell wall of Gram-positive bacteria is an approximately 20-50 nm thick, three dimensional net-like structure mainly composed of multiple layers of peptidoglycan, additional poly-anionic teichoic acids and substantial proportions of proteins. Together, they form a negatively charged matrix maintaining an optimal metal cation homeostasis essential to membrane-bound enzyme systems. Peptidoglycan strands of varying lengths consist of disaccharide subunits of N-acetyl glucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) with short stem peptides linked to carboxyl group of muramyl residues (Foster & Popham, 2002). The lipid linked disaccharide-pentapeptide precursors of peptidoglycan, called lipid II, are synthesized in the cytoplasm by a great number of enzymes. Lipid II precursors are polymerized into glycan strands and cross-linked to each other in enzymatic reactions carried out by penicillin-binding proteins (PBSs) present in the cell wall (Ghuysen, 1991). Rigid peptidoglycan strands are cross-linked to each other via flexible peptide bridges, thus forming a strong but nevertheless elastic structure.

Peptidoglycan and poly-anionic glycopolymers are present in the cell wall in almost equal amounts. For example, in *B. subtilis* glycopolymers make up 35-60% of the cell wall dry weight (Neuhaus & Baddiley, 2003). The structures of glycopolymers are often specific to a particular species or even strain, and are highly variable in their sugar content, net charge and "side group decorations". The most important group of glycopolymers is teichoic acids, which are either covalently linked to sugar residues of the peptidoglycan (wall teichoic acids, WTAs) or anchored to the cell membrane via a glycolipid anchor (lipoteichoic acids, LTAs) (Delcour *et al.*, 1999; Foster & Popham, 2002). The cell wall of Gram-positive bacteria usually contains one type of both teichoic acids, but there are also exceptions such as *B. subtilis*, which has one LTA, two kinds of WTAs and in addition can produce teichuronic acid under low-phosphate conditions.

Teichoic acids are linear polymers formed of sugar monomers linked via anionic phosphodiester linkages, which are responsible for the overall net negative charge of the cell surface. The sugar backbones of WTAs often contain glycerol or ribitol, but tetroses, hexoses and complex sugar combinations also exist. LTAs are usually less variable than WTAs and are mainly composed of glycerol-phophate repeating units (Archibald *et al.*, 1993; Neuhaus & Baddiley, 2003; Weidenmaier & Peschel, 2008). Most teichoic acids contain additional sugars or amino acids, such as D-alanine. The D-alanine content of teichoic acids is controlled by the *dlt* operon discussed in earlier sections. Teichuronic acids differ from classical teichoic acids in having a backbone lacking the phosphate groups. However, they are still anionic due to the presence of uronic acid, for example glucuronic acid in *B. subtilis* (Neuhaus & Baddiley, 2003; Weidenmaier & Peschel, 2008).

The function of teichoic acids has not been completely elucidated. They are considered to be essential for the viability of Gram-positive bacteria and proposed to have important roles in cell wall function, maintenance and turnover. However, studies with mutants lacking or having reduced amounts of certain teichoic acids have revealed that WTAs are dispensable, at least under laboratory conditions, in both *S. aureus* and *B. subtilis* (D'Elia *et al.*, 2006a; D'Elia *et al.*, 2006b). On the contrary, LTAs seem to be essential for *S. aureus*, but mutants with as much as a 90% reduction in the LTA content are still viable and do not show any major phenotypic changes (Fedtke *et al.*, 2007; Grundling & Schneewind, 2007). This does not exclude the fact that TAs have a fundamental role in maintaining overall cell integrity. In addition to their crucial role in protecting the cell envelope from harmful substances such as antibiotics, surfactants, phages, CAMPs and antimicrobial fatty acids of human skin, TAs have recently been shown to contribute to host cell adhesion and colonization, inflammation and immune activation caused by Gram-positive pathogens (Kohler *et al.*, 2009; Weidenmaier & Peschel, 2008; Xia *et al.*, 2010). Furthermore, it was recently shown that WTAs have a role in targeting the major autolysin Atl of *S. aureus* to the septal region of the cell surface leading to cell division (Schlag *et al.*, 2010)

Cell membrane

The cell membrane controls the permeability of the bacterial cell and the transportation of various kinds of molecules. In addition, it is a place where functions related to respiration, the replication of DNA and synthesis of cell wall components occur.

The composition of the prokaryotic cell membrane differs from that of eukaryotic cells. Bacterial membranes are typically enriched in negatively charged phospholipids such as phosphatidylglycerol, cardiolipin and phosphatidylserine, whereas the zwitterionic phospholipids phosphatidylcholine, phosphatidylethanolamine and sphingomyelin common in eukaryotic cells are rarely present in prokaryotic membranes. Sterols such as cholesterol and ergesterol are also absent from bacterial membranes (Yeaman & Yount, 2003). Due to these differences, bacterial membranes tend to be generally much more electronegative than eukaryotic membranes. However, Gram-positive bacteria can reduce the overall negative charge of cell membrane by producing positively charged lysine esters of phosphatidylglycerol, lysyl-PG (Peschel *et al.*, 2001). As in eukaryotic cells, the membrane lipids are asymmetrically distributed between the two lipid layers of the bacterial membrane, influencing membrane stability, intercellular recognition and signal transduction (Pomorski *et al.*, 2004; Tannert *et al.*, 2003). The lipid content varies between bacterial species. For example, in *B. subtilis*, phosphatidylglycerol accounts for 75% of the total membrane lipid content, the rest being mainly lysyl-PG and cardiolipin, whereas in *S. aureus* up to 38% of membrane lipids are lysyl-PG (Peschel *et al.*, 2001).

2.3.2 Alternative sigma factors

Adaptation to environmental changes demands timely regulation of gene expression and only optimal sets of genes are transcribed under specific conditions. The binding of a sigma factor to the core RNA polymerase is essential for the recognition of the appropriate promoter sequence and the initiaton of transcription. The promoter selectivity of RNA polymerase can be altered by alternative σ factors that are activated by adequate triggers (Paget & Helmann, 2003). All bacteria have one primary σ factor similar to σ^{70} in *E. coli* and σ^{A} in *B. subtilis* mediating most of the transcription in exponentially growing cells. The number of σ factors varies between bacterial species; for example, *E. coli* posses six σ factors, *B. subtilis* at least 16 σ factors and *Streptomyces coelicolor* over 60 σ factors, while only four σ factors are found from the genome of *S. aureus* (Helmann, 2006; Shaw *et al.*, 2008). One of the best characterized alternative σ factors is σ^{B}, which orchestrates the general stress responses of Gram-positive bacteria such as *B. subtilis* and *S. aureus* (Hecker & Volker, 2001; Morikawa *et al.*, 2003).

Sigma factors are classified into two major families known as the σ^{70} and σ^{54} families (Gross *et al.*, 1998; Gruber & Gross, 2003; Helmann & Chamberlin, 1988). Most σ factors belong to the σ^{70} family, which is further divided into groups based on sequence similarity and domain topology. These groups include essential primary σ factors and their non-essential paralogues, σ factors contributing to motility,

sporulation and heat shock resistance and the **e**xtra**c**ytoplasmic **f**unction (ECF) σ factors, which are the largest group in the σ^{70} family (Gruber & Gross, 2003; Staron *et al.*, 2009).

ECF σ factors are often involved in controlling cell envelope stress responses or transport processes (Helmann, 2002; Lonetto *et al.*, 1994). Comparative genomic analyses have revealed that ECF σ factors are ubiquitously present in bacterial species. Although there is an average of six ECF σ factors per bacterial genome (Staron *et al.*, 2009), the number of ECF σ factors varies greatly between species. For example, *Streptomyces coelicolor* is predicted to encode more than 50 ECF σ factors, the other extreme being *S. aureus*, with only a single putative ECF σ factor (σ^S) so far characterized (Helmann, 2006; Shaw *et al.*, 2008).

ECF σ factors share several common features. Unlike the primary σ factors binding the classical consensus sequences in the -10 ('TATAAT') and -35 ('TTGACA') regions (Helmann, 1995; Travers, 1987), many of the ECF σ factors recognize a highly conservative 'AAC' motif in the -35 region and 'CGT' clusters in the -10 region (Helmann, 2002; Lane & Darst, 2006). ECF σ factors also lack two of the four conserved domains present in the primary σ factors. In addition, ECF σ factors can autoregulate their own expression and are usually cotranscribed with their cognate anti-σ factors. Anti-σ factors are transmembrane proteins that bind and inactivate σ factors in the absence of an appropriate stimulus. After receiving an adequate signal, anti-σ factor is released from the σ factor, enabling the activation of RNA polymerase by ECF σ factor (Helmann, 2002; Jordan *et al.*, 2008; Staron *et al.*, 2009).

ECF sigma factors of *B. subtilis*

The *B. subtilis* genome comprises seven ECF σ factors, σ^W, σ^M, σ^X, σ^Y, σ^V, σ^Z and σ^{YlaC} (Helmann, 2002; Kunst *et al.*, 1997). At least σ^W, σ^M, σ^X have been shown to contribute to cell envelope stress responses.

The best characterized ECF σ factor in *B. subtilis* is σ^W. Based on promoter consensus searches, about 30 target promoters of σ^W controlling the expression of about 60 genes have been identified (Cao *et al.*, 2002a; Huang *et al.*, 1999). The σ^W regulon is induced by numerous stress conditions, including cell wall active antibiotics such as vancomysin, cephalosporin, fosfomycin and D-cycloserine, as well as membrane active compounds, alkali shock and bacteriocins (Butcher & Helmann, 2006; Cao *et al.*, 2002b; Wiegert *et al.*, 2001). It is postulated that σ^W regulates genes involved in the inactivation or extrusion of toxic compounds from the cell (Helmann, 2006). The role of σ^W in antibiotic resistance was confirmed in studies demonstrating that σ^W deletion causes increased sensitivity to fosfomycin and antimicrobial compounds synthesized by other *Bacillus* species (Butcher & Helmann, 2006; Cao *et al.*, 2001).

The σ^W-encoding gene (*sigW*) is cotranscribed with its cognate anti-σ factor gene *rsiW*. In the absence of a proper signal, the membrane-anchored RsiW directly binds

to σ^W. Activation of σ^W is achieved through a proteolytic cascade releasing σ^W from RsiW. The first step of the cascade is degradation of the extracytoplasmic domain of RsiW by a membrane-bound protease, PrsW (Ellermeier & Losick, 2006; Heinrich & Wiegert, 2006). This is followed by the RasP-mediated cleavage of the membrane spanning region of RsiW, leading to the formation of a soluble N-terminal fragment of RsiW (Schobel *et al.*, 2004). This fragment is further degraded by the cytoplasmic ClpX proteolytic complex (Zellmeier *et al.*, 2006).

ECF σ factor σ^M directly controls the expression of at least 57 genes. In addition, σ^M activates several other gene regulators. The functions of these σ^M regulated genes include cell wall synthesis, cell division and the determination of cell shape, DNA repair and detoxification (Eiamphungporn & Helmann, 2008). The σ^M regulon is activated by several stress conditions such as an acidic pH, high salinity, heat, superoxide, paraquat and cell wall active antibiotics (Cao & Helmann, 2002; Horsburgh & Moir, 1999; Mascher *et al.*, 2003; Minnig *et al.*, 2003; Thackray & Moir, 2003). Mutants with a σ^M deletion display increased sensitivity to certain cell envelope active compounds such as bacitracin, moenomycin, SDS and some β-lactams (Cao & Helmann, 2002; Mascher *et al.*, 2007). σ^M is also activated under phosphate deprivation and involved in TA synthesis in the *B. subtilis* strain W23 (Minnig *et al.*, 2005). σ^M is cotranscribed with its anti-σ complex composed of two separate proteins, YhdL and YhdK. The direct binding of σ^M to the N-terminal part of YhdL has been demonstrated by using a yeast two-hybrid system (Yoshimura *et al.*, 2004).

The first ECF σ factor of *B. subtilis* characterized in detail was σ^X. It is cotranscribed with the gene *rsiX* encoding the anti-σ factor of σ^X (Huang & Helmann, 1998). The σ^X regulon comprises ~30 genes and the main role of σ^X is predicted to be in modulation of the net negative charge of the cell envelope (Cao & Helmann, 2004). This is carried out by two σ^X regulated operons, *dltABCDE* and *pssA-ybfM-psd*. The former operon is responsible for the D-alanylation of wall TAs (Perego *et al.*, 1995) and the latter is involved in the synthesis of the neutral lipid phosphatidylethanolamine, resulting in a lower net negative charge of the cell envelope (Cao & Helmann, 2004).

The role of the other ECF σ factors, σ^Y, σ^V, σ^Z and σ^{YlaC}, remains unclear. The σ^Y regulon consists of six genes organized into one cistron. This cistron contains a gene predicted to encode a toxic peptide and another gene encoding a putative immunity protein against toxic peptide (Cao *et al.*, 2003; Tojo *et al.*, 2003). It was also demonstrated that disruption of σ^Y might have an effect on sporulation induced by nitrogen depletion (Tojo *et al.*, 2003). DNA macroarray analysis of genes regulated by σ^V revealed 13 genes belonging to the σ^V regulon, although an extensive overlap between other ECF σ factors complicates the defining of the regulons (Zellmeier *et al.*, 2005).

Although the role of ECF σ factors in the maintenance of cell envelope integrity under stress conditions seems to be indisputable, little is known about the exact signals activating the σ factors and how these signals are received. Studies with *E.*

coli have revealed that unfolded proteins in the periplasm trigger the proteolytic degradation of the anti-σ factor of σ^E, leading to the activation of σ^E (Rowley *et al.*, 2006). These observations suggest that the primary candidate for the sensory module of the ECF σ factors is the protease starting the proteolytic cascade of anti-σ factors, e.g. PrsW in the case of σ^W of *B. subtilis* (Helmann, 2006; Jordan *et al.*, 2008). Furthermore, an extensive regulatory overlap between the different ECF σ factors has been demonstrated in several studies both *in vitro* and *in vivo*. Many of the operons regulated by ECF σ factors contain multiple promoter elements recognized by several ECF σ factors (Cao & Helmann, 2002; Huang *et al.*, 1998; Mascher *et al.*, 2007; Qiu & Helmann, 2001).

2.3.3 Two-component systems

Two-component signal transduction systems are one of the most important bacterial sensory systems for obtaining information from the world outside. TCSs consist of two functional units, a membrane-anchored sensor histidine kinase (HK) sensing extracellular signals and a cytoplasmic response regulator (RR) responsible for mediating differential gene expression (Fig. 5). HKs are typically integral membrane proteins containing two distinct domains: a highly variable N-terminal input domain capable of binding a specific signalling molecule or reacting to a physical stimulus and a conserved C-terminal cytoplasmic transmitter domain interacting with the cognate RR. The transmitter domain is responsible for autokinase and ATP-binding activities of HK. The activation of TCS includes three phosphoryl-transfer reactions. First, binding of the signal ligand causes an ATP-dependent autophosphorylation reaction of a conserved histidine residue in the transmitter domain of HK. Secondly, a phosphoryl group is transmitted further to an acceptor aspartic acid residue in the N-terminal receiver domain of RR, leading to activation of the C-terminal output domain with DNA-binding properties (Parkinson, 1993; Stephenson & Hoch, 2002). Finally, RR is dephosphorylated in order to set the system back to an inactive state (Parkinson, 1993).

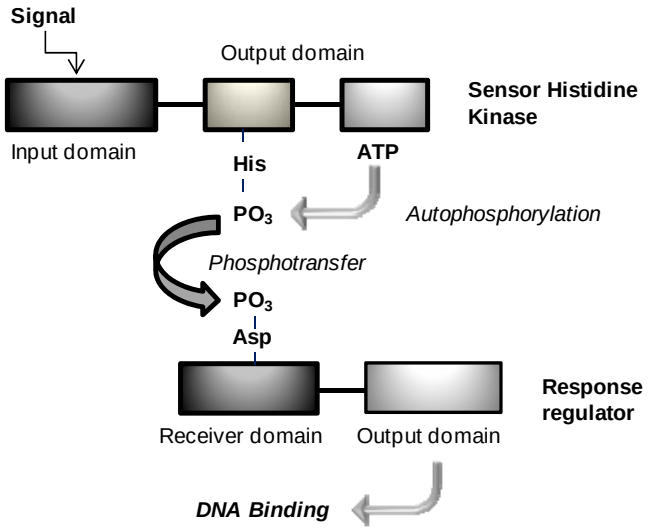

Figure 5. Schematic representation of the two-component signal transduction system. Adapted from Stephenson and Hoch (2002).

TCSs are ubiquitously distributed among bacterial species and average bacterial genome harbors 50 genes encoding TCSs (Whitworth, 2008). Early attempts to classify histidine kinases have mainly been based on sequence analysis of the conserved regions of the transmitter domains, providing only scarce information on the functional role of the different HKs (Fabret *et al.*, 1999; Grebe & Stock, 1999). Comparisons of the input domains of HKs have been problematic due to the great sequence variation in these domains, reflecting the huge variety of environmental stimuli sensed by bacterial cells (Hoch, 2000). It seems that more precise predictions about the physiological roles of individual TCS can be gained by comparing the domain architecture and genomic context of input domains of HKs instead of mere similarities in sequences (Mascher, 2006; Mascher *et al.*, 2006).

Based on HK topology, most TCSs can be classified into three major groups (Mascher *et al.*, 2006). The largest group comprises TCSs with extracellular sensing HKs having an extracellular sensory domain flanked by at least two transmembrane helices. This kind of extracellular domain stucture is typical for HKs sensing solutes and nutrients such as nitrate, nitrite or citrate. In these cases, signal detection occurs by direct interaction between the input domain and the molecule (Janausch *et al.*, 2002; Stewart, 2003; Stewart & Bledsoe, 2003). The group of extracellular sensing HKs also includes two-component systems mediating resistance to cell envelope stress, the best characterized example being PhoQ and its cognate RR PhoP in the Gram-negative bacterium *Salmonella typhimurium* (Groisman *et al.*, 1989; Miller *et al.*, 1989). The PhoQP TCS confers resistance to CAMPs and is activated by direct binding of antimicrobial peptides to the extracellular sensor domain, leading to modifications of lipopolysaccharides and lipid A on the cell surface (Bader *et al.*,

2005). This group of HKs comprises several kinases associated with β-lactam resistance in Gram-positive bacteria such as CroS in *Enterococcus faecalis*, CesK and LisK in *Listeria monocytogenes* and CiaH in *Streptococcus pneumoniae* (Jordan *et al.*, 2008).

The second largest group of HKs consists of cytoplasmic-sensing HKs, either being membrane anchored or soluble proteins. The input domain of these HKs locates in the cytoplasm and therefore senses changes in intracellular conditions. The third diverse group of HKs includes sensor kinases with 2 to 20 transmembrane regions connected by very short extracellular linkers buried in the cell membrane (Mascher, 2006; Mascher *et al.*, 2006). In other words, they lack almost the entire extracellular sensing domain, leading to an assumption that they sense stimuli present in the membrane or in the membrane interface. The transmembrane helices seem to be important for signal recognition. These TCSs participate in various physiological processes such as thermosensing, quorum sensing, energy metabolism and solute transport. This group of HKs includes the subgroup of intramembrane-sensing HKs playing a key role in the sensing of cell envelope stress in Gram-positive bacteria (Jordan *et al.*, 2008). The sensory domain organization of these small HKs is strikingly similar, comprising two transmembrane helices connected by short extracytoplasmic loops less than 25 aa in length. Two types of genomic organization can be distinguished among these HKs: kinases that are parts of three-component systems and kinases that are linked to ABC transporters (such as LiaS and BceS, respectively, in *B. subtilis*) (Mascher, 2006). A similar domain composition of the input domain is also found in other phylogenetically distinct cell wall stress-related TCSs, such as VanRS responsible for inducible resistance to vancomycin (Hong *et al.*, 2004; Hutchings *et al.*, 2004) and other closely related glycopeptides in *Streptomyces coelicolor*, and PmrB/BasS-like HKs contributing to CAMP resistance in *E. coli* and *Salmonella* spp. (Gunn *et al.*, 1998; Gunn *et al.*, 2000; Wosten & Groisman, 1999).

TCSs in *B. subtilis*

The genome of *B. subtilis* harbors 36 HKs and 34 RRs (Fabret & Hoch, 1998; Kunst *et al.*, 1997) listed in Table 1. The TCSs involved in cell envelope stress tolerance include LiaRS, BceRS and its homologues, WalKR (YycFG) and CssRS.

Table 1. Two-component systems in B. subtilis.

Kinase	Response regulator	Function
BceS	BceR	Resistance to antibiotics affecting the cell envelope
CheA	CheY	Chemotaxis and motility
CitS	CitT	Mg^2/citrate transport
ComP	ComA	Early competence
CssS	CssR	Protein quality control
DctS	DctR	Transport of fumarate and succinate
DegS	DegU	Regulation of production of degradative enzymes and competence
DesK	DesR	Adaption to cold temperature
GlnJ	GlnL	Glutamine degradation
KinA	Spo0F	Initiation of sporulation
KinB	Spo0F	Initiation of sporulation
KinC		Initiation of sporulation
KinD	Spo0A-P	Initiation of sporulation
KinE	Spo0F-P	Initiation of sporulation
LiaR	LiaR	Unknown
LytS	LytT	Rate of autolysis
NatK	NatR	Sodium ion extrusion
PhoR	PhoP	Phosphate starvation
ResE	ResD	Aerobic and anaerobic respiration
YbdK	YbdJ	Unknown
YcbM	YcbL	Unknown
YclK	YclJ	Anaerobic growth
YdfH	YdfI	Unknown
YesM	YesN	Unknown
YfiJ	YfiK	Regulation of amino acid biosynthesis and uptake
YhcY	YhcZ	Unknown
YkoH	YkoG	Oxidative stress
YrkQ	YrkP	Unknown
YufL	YufM	Transport of malate
YvcQ	YvcP	Involved in bacitracin resistance
YvfT	YvfU	Unknown
YvrG	YvrH	Involved in membrane conformation and lipid composition
YwpD		Unknown
YxdK	YxdJ	Responds to CAMPs
YxjM	YxjL	Unknown
YycG (WalK)	YycF (WalR)	Essential functions, cell wall processes

LiaRS in *B. subtilis* has been reported to strongly respond to several cell wall active antibiotics such as bacitracin, ramoplanin and vancomycin (Mascher *et al.*, 2003; Mascher *et al.*, 2004). It is also induced by other stress-causing conditions including alkaline shock, secretion stress, ethanol, phenol and organic solvents, although clearly to a lesser extent (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Tam le *et al.*, 2006; Wiegert *et al.*, 2001). LiaRS is also activated by the intrinsic peptide produced by the *yydFGHIJ* operon of *B. subtilis* (Butcher *et al.*, 2007). The LiaRS locus comprises 6 genes, *liaIH-liaGFSR* with two promoter regions upstream from *liaI* and *liaG*. *liaGFSR* encodes the histidine kinase LiaS and its cognate response regulator LiaR, whereas LiaF is an inhibitor of LiaRS, binding directly to LiaR (Jordan *et al.*, 2006; Mascher *et al.*, 2003). This is why LiaRSF is often referred to as a three-component system. The gene *liaG* encodes a putative membrane-anchored protein with unknown function. The *liaGFSR* genes are constitutively expressed at a low level from the promoter upstream from *liaG*. In

contrast, *liaIH* genes are completely silent in the absence of LiaR. LiaRS probably controls the expression of only two operons, *liaIH-GFSR* itself and *yhcYZ-yhdA*, the primary target being *liaIH-GFSR* (Jordan *et al.*, 2006). The function of proteins encoded by LiaIH is largely unknown; LiaI is a small hydrophobic protein with two putative transmembrane helices and LiaH belongs to the phage shock protein family. The expression of *liaIH-GFSR* operon is strictly controlled by at least five regulators, and without any extracellular signal it is kept inactive during the exponential growth phase (Jordan *et al.*, 2006; Jordan *et al.*, 2007). However, it is activated at the onset of the stationary phase, when bacterial cells are adapting to worsening living conditions, finally leading to the formation of dormant endospores (Jordan *et al.*, 2007).

Three intramembrane sensing TCSs are found from the genome of *B. subtilis*: *bceRS-bceAB* (formerly *ytsAB-ytsCD*), *yvcPQ-yvcRS* and *yxdJK-yxdLM* (Joseph *et al.*, 2002; Joseph *et al.*, 2004). Typical of them all is that TCS encoding genes are located in the immediate upstream region of the genes encoding ABC transporters. Both TCS genes and ABC transporter genes are organized into separate operons with their own promoter regions. TCS genes are expressed constitutively, whereas the genes encoding ABC transporters are under the control of the cognate TCS (Mascher, 2006). The BceRS-BceAB system seems to play a key role in bacitracin resistance in *B. subtilis*. The recognition of sublethal concentrations of bacitracin by BceRS causes the expression of the detoxification unit encoded by *bceAB* responsible for the removal of bacitracin from the cells (Mascher *et al.*, 2003; Ohki *et al.*, 2003). The two other systems, YxdJK-YxdLM and YvcPQ-YvcRS, are poorly characterized, but it seems that YvcRS might have a role in lantibiotic resistance; hence, it is activated by nisin and to a lesser extent by bacitracin (Jordan *et al.*, 2008; Mascher *et al.*, 2003). Despite genetic analysis, the function of the YxdJK-YxdLM system has remained unclear (Joseph *et al.*, 2004).

The YycFG system is the only TCS essential for the viability of *B. subtilis* (Fabret & Hoch, 1998). YycFG is a part of the operon *yycFGHIJ*, in which the *yycH* and *yycI* encode inhibitors of sensor kinase YycG (Szurmant *et al.*, 2005; Szurmant *et al.*, 2007). The YycFG regulon consists of several genes involved in cell wall metabolism and homeostasis (Bisicchia *et al.*, 2007; Dubrac & Msadek, 2004). It seems that YycFG senses some signal derived from normal cell wall metabolism, since the TCS is active under normal growth conditions. The activating signal may be the D-ala-D-ala moiety of lipid II (Dubrac *et al.*, 2008). This hypothesis is further supported by the finding that the YycFG regulon is not differentially expressed under cell envelope stress caused by vancomycin (Bisicchia *et al.*, 2007).

CssRS responds to cell envelope stress caused by the accumulation of misfolded proteins at the membrane-cell wall interface (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2001). CssRS is a functional orthologue of the Cpx system of *E. coli* (Connolly *et al.*, 1997; Danese & Silhavy, 1997; Missiakas & Raina, 1997; Pogliano *et al.*, 1997) and it determines the level of proteolytic activity in the cell envelope by controlling the expression of *htrA* and *htrB* genes encoding serine-type surface proteases involved in protein quality control (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2001).

TCSs in *S. aureus*

The *S. aureus* genome consists of 17 potential TCSs (Kuroda *et al.*, 2001) listed in Table 2. TCSs reported to participate in cell envelope stress responses are VraSR, GraRS, YycFG and LytSR.

Table 2. Two-component systems in S. aureus

Kinase	Response regulator	Function
AgrC (SA1843)	AgrA (SA1844)	Quorum sensing, regulation of virulence factors
ArlS (SA1246)	ArlR (SA1247)	Virulence, regulation of exoprotein production
GraS (SA0614)	GraR (SA0614)	CAMP resistance, vancomycin resistance
KdpD (SA1882)	KdpE (SA1883)	Potassium transport
LytS (SA0250)	LytR (SA0251)	Regulation of autolysis
NreB (SA2180)	NreC (SA2179)	Regulation of nitrite/nitrate reduction
PhoR (SA1515)	PhoP (SA1516)	Regulation of phosphatase synthesis
SA0216	SA0215	Unknown
SA1158	SA1159	DesK homologue, adaptation to low temperature
SA1666	SA1667	Unknown
SA2152	SA2151	Heme sensor system
SA2417	SA2418	Unknown
SaeS (SA0660)	SaeR (SA0661)	Virulence, regulation of toxin production
SrrB (SA1322)	SrrA (SA1323)	Respiratory response regulator, virulence
VraS (SA1701)	VraR (SA1700)	Antibiotic resistance, PG synthesis
WalK (SA0017)	WalR (SA0018)	Cell wall and membrane composition, essential

VraSR is a LiaRS homologue originally discovered to be upregulated in a vancomycin-resistant strain of *S. aureus*, and later on also in a vancomycin intermediate-resistant strain (Kuroda *et al.*, 2000; McAleese *et al.*, 2006). The *vraSR* genes are located in the same operon with two upstream genes, one encoding a LiaF homologue and another one encoding a putative protein with unknown function. Unlike LiaRS, the VraSR regulon comprises several genes having a role in cell envelope synthesis and maintenance. The VraSR regulon is induced by cell wall interfering antibiotics such as vancomycin, β-lactams, bacitracin, teicoplanin and D-cycloserine. Deletion mutants of VraSR display increased sensitivity to all inducing antibiotics, excluding fosfomycin and D-cycloserine (Kuroda *et al.*, 2003). VraSR is also activated by perturbations in cell wall assembly (Gardete *et al.*, 2006; Sobral *et al.*, 2007), but not by general sress conditions such as osmotic shock, heat or pH changes (Kuroda *et al.*, 2003).

GraRS is homologous to BceRS in *B. subtilis* and it controls the expression of its downstream genes encoding an ABC transporter, VraFG. In addition, GraRS, also referred as Aps, controls the expression of *dlt*, *mprF* and *vraDE* operons (Li *et al.*, 2007a). GraRS mediates resistance to several CAMPs, including LL-37, gallidermin, polymyxin B and the LP9 peptide derived from human lysozyme (Herbert *et al.*, 2007; Kraus & Peschel, 2008; Meehl *et al.*, 2007). In addition, GraRS mediates vancomycin-intermediate resistance in *S. aureus* (Herbert *et al.*, 2007; Howden *et al.*, 2008). Deletion of *graRS* leads to alterations in the charge of the cell envelope and attenuated virulence (Kraus & Peschel, 2008; Li *et al.*, 2007a).

Like its homologue in *B. subtilis*, YycFG of *S. aureus* is essential for the viability of bacterial cells (Martin *et al.*, 1999). YycFG seems to be a positive regulator of

autolytic activity. YycG-depleted cells have shown changes in their cell wall structure, including an increased glycan chain length and peptidoglycan cross-linking. Due to the importance of YycFG in cell wall metabolism, it was renamed as WalKR (Dubrac *et al.*, 2007). It has also been demonstrated that up-regulation of YycFG confers decreased susceptibility to vancomycin and daptomycin (Friedman *et al.*, 2006; Jansen *et al.*, 2007).

LytSR has been identified to contribute to the regulation of autolysis and murein hydrolase activity in the cell wall (Brunskill & Bayles, 1996). It has been suggested that LytRS senses changes in the membrane potential caused by proton motive force (Patton *et al.*, 2006).

3 Aims of the study

Bacterial cells are amazingly adaptable to changing environmental conditions. The aim of this thesis was to learn more about the stress responses caused by cationic antimicrobial peptides attacking the cell membrane essential for the survival of bacterial cells. These toxic peptides are commonly present in various bacterial habitats and are produced either by other microorganisms competing for the living space or by host cells defending themselves against bacterial invasion. Understanding the mechanisms by which bacteria can resist toxic compounds may help in the development of new antimicrobial drugs in the future.

The specific aims of the study were:

1) To investigate the stress responses caused by different types of cationic antimicrobial peptides in two different Gram-positive bacteria, *B. subtilis* and *S. aureus*.

2) To further characterize role of the highly induced genes in CAMP stress. In particular, the genes participating in cell signalling, ECF σ factors and two-component systems, were subjects of interest.

3) To study the effect of the cell envelope charge on the function of TCSs mediating cell envelope stress.

4) To determine whether transcriptome analysis of bacterial cells treated with CAMPs could reveal something about the action mechanism of peptides.

4 Materials and methods

The bacterial strains and plasmids used in this study are listed in the original articles. Methods and CAMPs used in this study are listed in Tables 3 and 4, respectively.

Table 3. Methods used in this study

Method	Described and used in
Antimicrobial susceptibility tests	I, III
β-Lactamase assay	II
cDNA synthesis	I, II, III
2D electrophoresis	I, II
DNA macroarray	I
Molecular cloning techniques	I, II, III
Oligo DNA microarray	III
Phenotype array	III
Quantitative real-time RT-PCR	I, II, III
RNA isolation	I, II, III
Site-directed mutagenesis	I, II, III

Table 4. Cationic antimicrobial peptides used in transcriptome analyses

Peptide	Sequence	Charge	Origin
Dermaseptin K4-S4(1-16)	ALWKTLLKKVLKAAK-NH_2	+5	Amphibian
LL-37	LLGDFFRKSKEKIGKEFKRI VQRIKDFLLRNLVPRTES-NH_2	+6	Human
Ovispirin-1	KNLRRIIRKIIHIIKKYG-NH_2	+8	Ovine
Protegrin 1	RGGRLCYCRRRFCVCVGR-NH_2	+6	Porcine
Temporin L	FVQWFSKFLGRIL-NH_2	+2	Amphibian
Poly-L-lysine	L-lys_n	+n	Synthetic

5 Results and discussion

5.1 CAMPs induce a strong stress response in bacterial cells (I, III)

5.1.1 CAMP stress response in *Bacillus subtilis* (I)

In order to investigate the stress responses caused by CAMPs in *B. subtilis*, three different peptides were chosen for the transcriptome analysis. Human cathelicidin LL-37 is a linear α-helical peptide, whereas porcine protegrin PG-1 is a cysteine rich peptide forming a β-sheet hairpin structure stabilized by two disulphide bridges (Johansson *et al.*, 1998; Kokryakov *et al.*, 1993). A synthethic peptide, poly-L-lysine (PLL), was selected as a synthetic analogue of cationic peptides (Vaara & Vaara, 1983), although it differs from natural peptides in being a non-amphipathic molecule. LL-37 is predicted to disrupt membranes either by a carpet mechanism or by forming toroidal pores (Henzler-Wildman *et al.*, 2004; Henzler Wildman *et al.*, 2003; Oren *et al.*, 1999). However, it has also recently been suggested that LL-37 may exert its antimicrobial effects by compromising the membrane barrier properties of the target microbes by a mechanism involving cytotoxic oligomers forming amyloid-like fibres (Sood *et al.*, 2008). PG-1 causes rapid lysis of bacterial cells by forming pore-like oligomeric structures known as β-barrels (Mani *et al.*, 2006; Tang & Hong, 2009). The mechanism of action of PLL on membranes is still unclear.

Samples for transcriptome analysis were collected from bacterial cultures treated with sublethal concentrations of CAMPs and were compared with control samples without peptide treatment. The peptides were added at the exponential growth phase and the samples were collected 20 min after the addition of peptides. The amount of peptide needed to cause a slight growth arrest in cell cultures without killing the bacterial cells varied between peptides from a nanomolar concentration of PG-1 to a millimolar concentration of PLL. Both the CAMP-treated and control samples were treated similarly. The purified total RNA was converted to cDNA labelled with P^{33} and hybridized on DNA macroarrays containing all ~4000 ORFs of the *B. subtilis* genome. qRT-PCR was also used to verify some of the most interesting gene expression responses.

As expected, peptides had a strong impact on the gene expression of treated cells. Only the induced genes were analysed further. All genes induced at least two-fold in two independent array experiments were considered as upregulated ones. Altogether, LL-37 activated 96 genes, the induction ratios varying from 2-fold to 60-fold. LL-37 seemed to cause a more severe stress reaction than the other natural peptide PG-1, which caused activation of 58 genes with clearly lower induction

ratios (from 2-fold to 15-fold) than LL-37. The synthetic analogue PLL caused the upregulation of 86 genes (from 2- to 18-fold induction) (I).

The fact that most of the genes responding to CAMP stress were genes with an unknown function made interpretation of the results difficult, but some common features could be identified from the induction patterns of all three peptides. One of the most prominent findings was the activation of genes belonging to the σ^W and σ^M regulons (I, Tables 5 and 6). These two ECF σ factors are predicted to have a role in the maintenance of cell envelope integrity and are activated by several stress conditions such as alkaline shock and antibiotics, interfering peptidoglycan synthesis (Cao *et al.*, 2002b; Wiegert *et al.*, 2001). Genes that were induced by all three peptide treatments belonged to at least one of the ECF σ factor regulons, and many of these genes participate in interactions with cell envelope-interfering compounds (I). Such genes included the *dlt* operon responsible for the D-alanylation of lipoteichoic and wall teichoic acids (Perego *et al.*, 1995) contributing to increased resistance to antimicrobial peptides (Cao & Helmann, 2004; Peschel *et al.*, 1999), and *bcrC* encoding an alternative undecaprenyl pyrophosphate phosphatase related to bacitracin resistance (Bernard *et al.*, 2005; Cao & Helmann, 2002). Interestingly, only some of the genes regulated by these σ factors were induced. Of the 30 verified promoters controlling the expression of about 60 genes of the σ^W regulon (Cao *et al.*, 2002a; Huang *et al.*, 1999), about one third were induced by the natural peptides LL-37 and PG-1. PLL was the most effective activator of the σ^W regulon and induced 23 out of the 30 verified promoters known to belong to this regulon (I). The same phenomenon was also seen in the case of σ^M-regulated genes, although LL-37 seemed to be a more effective activator of σ^M than the other two peptides tested (I).

Table 5. Induction of the σ^W regulon by CAMPs

Category/Operons*	Function or Homology	Upregulation by LL-37	PG-1	PLL
Regulation				
sigW rsiW	SigW and its anti-σ factor		+	+
ysdB	Negative regulator of SigW			
Cell envelope synthesis				
pbpE	Penicillin binding protein	+	+	+
racX	Amino acid racemase	+	+	+
yuaFGI (?)	YuaG: similar to flotillin	+	+	+
Resistance to toxic peptides				
fosB	Fosfomycin resistance			
yfhLM	SdpC resistance		+	+
yknWXYZ	SdpC resistance		+	+
YqeZyqfAB	Sublancin resistance	+	+	+
ydbST	Resistance to *B. amyloliquefaciens*		+	+
Predicted proteases				
yjoB	ATPase possibly involved in protein degradation			+
yteIJ	Putative integral inner membrane protein			
Detoxification (?)				
ybfO	Putative exported hydrolase			
yceC	Putative stress adaptation protein	+	+	+
ydjP	Putative peroxydase			
ythPQ	Putative ABC transporter			+
Small peptides (bacteriocins?)				
ydjO				
yvlC				
yxzE				
yoaF				+
yoaG				

*Possible functional roles based on sequence similarities are indicated by question marks.
σ^W-dependent genes with an unknown function are not listed. Categories of genes adapted from Helmann 2006.

Table 6. Induction of the SigM regulon by CAMPs

Category/Operon	Function or Homology	Upregulation by LL-37	PG-1	PLL
Regulation				
sigM *yhdLK**	SigM and its anti-σ factor	+		+
yjbCspx	Spx transcription factor	+	+	+
abh	Transition state regulator		+	
ywaC	Putative ppGpp synthase			+
rapD	Response regulator aspartate phosphatase			
ywtF	Transcription factor			
Cell division/shape				
divIC	Component of septosome	+		
*(**murG**)B divIB ylxXW spb**	Cell division	+	+	
rodA	Cell division membrane protein			
*(maf)**ysxA**mreBCD**minCD**	Cell division and shape determination	+		
Cell envelope synthesis				
yfnI	Similar to lipoteichoic acid synthase			
bcrC	Undecaprenyl pyrophosphate (UPP) Phosphatase	+	+	+
*(**ydbO**-ydbP(as))-ddlmurF**	MurF: D-ala-D-ala ligase	+		
*recU**ponA**	PG synthesis, ponA: penicillin-binding protein	+		+
dltABCDE	D-alanylation of teichoic acids	+	+	
pbpX	Low MW penicillin-binding protein		+	
DNA monitoring and repair				
(sms)disA yacLM	DisA: DNA integrity monitoring protein			
Detoxification				
yrhHIJ	YrhH:putative methyltransferase, YrhJ:cytochrome P450	+		+
yqjL	Hydrolase, paraquat resistance			
yceCDEFG	Tellurium resistance operon	+	+	+

*The whole operon was not induced. The induced genes are marked with bold letters.
σ^M-dependent genes with an unknown function are not listed. Categories of genes adapted from Eiamphungporn and Helmann 2008.

On the other hand, several induced genes did not belong to any of the ECF sigma factor regulons (I). These results suggested that other regulators of transcription also mediate the stress response caused by CAMPs. The most strongly induced genes included *liaI* (58-fold induction by LL-37 and 15-fold induction by PG-1) and other genes of the *lia* operon and *yxdL* (22-fold induction by LL-37), which was strongly and specifically induced by LL-37 (I). Both *liaI* and *yxdL* encode proteins with an unknown function (see below). Neither of these operons is regulated by ECF σ factors. However, both operons are controlled by two-component systems adjacent to genes they are regulating. Taken together, these observations suggest that antimicrobial peptides elicit a complex stress response in *B. subtilis*, with several cell signalling systems involved.

Differences also existed in the stress responses activated by different peptides, in particular between natural peptides and PLL. For example, genes involved in purine, pyrimidine and ribosomal protein synthesis were strongly induced in cells treated with PLL, a phenomenon not seen with the natural peptides. Furthermore, some genes that were induced at high levels by either or both of the natural peptides were not activated by PLL as it was the case with the *lia* operon (I).

5.1.2 CAMP stress response in *Staphylococcus aureus* (III)

S. aureus is relatively resistant to CAMPs produced by mammalian cells. In this study, transcriptome analysis was used to determine whether certain special features exist in the CAMP stress response due to the pathogenic nature of *S. aureus*. The experimental setting was similar to that used in the transcriptome analysis of *B. subtilis*. All peptides used in the experiments were well-characterized linear α-helical peptides. This allowed us to determine whether strong peptide-specific responses or more general stress responses are triggered by all α-helical peptides in general. Ovispirin-1 is a derivative of ovine cathelicidin SMAP-29, whereas temporin L and dermaseptin S4 are expressed on the skin of amphibians (Navon-Venezia *et al.*, 2002; Sawai *et al.*, 2002; Simmaco *et al.*, 1996). A truncated derivative of S4 known as dermaseptin K4-S4(1-16) was used in this study (Navon-Venezia *et al.*, 2002). Temporin L is predicted to be a pore-forming peptide, whereas the other two peptides are predicted to act in a carpet-like manner (Brogden, 2005; Rinaldi *et al.*, 2002; Zhao & Kinnunen, 2002; Zhao *et al.*, 2002). All three peptides were C-terminally amidated in order to prevent possible proteolytic degradation. In addition, the effect of human cathelicidin LL-37 on gene expression of *S. aureus* was studied.

In order to capture the early stages of the stress response, samples were collected 10 min after peptide treatment. Peptides were added at sublethal concentrations causing a slight growth arrest, the exception being LL-37, as it turned out that *S. aureus* strain Newman was highly resistant to LL-37. The peptide concentrations used in array experiments were: 3 μM temporin L, 4 μM ovispirin-1, 3 μM dermaseptin K4-S4(1-16) and 3 μM LL-37. The changes in gene expression were analyzed using whole genome oligoarrays with Cy5- and Cy3-labelled cDNA (Charbonnier *et al.*, 2005). Three independent array experiments were performed. The results were verified by qRT-PCR in the case of the most interesting genes.

All three α-helical peptides caused induction of a large number of genes, most of which were upregulated by more than one peptide. Temporin L and ovispirin-1 caused the strongest effects on gene expression and upregulated 247 and 241 genes, respectively. Up to 40-fold induction ratios were detected with these two peptides. Dermaseptin K4-S4(1-16) induced 63 genes (from 2-fold to 17-fold induction) (III). LL-37 induced the expression of only 27 genes with low induction ratios, possibly reflecting lower stress in the LL-37-treated cells compared to cells treated with other peptides (Table 7). All α-helical peptides studied seemed to cause a similar kind of transcriptional response, regardless of the origin of the peptides. No significant differences were detected between the pore-forming and carpet-forming peptides, indicating that the mechanism of membrane dysfunction does not play a major role in the stress response. The induction patterns of CAMPs were strikingly similar to those observed in transcription profiling studies with vancomycin-treated cells (Kuroda *et al.*, 2003). A prominent feature was the induction of the VraSR two-component system, and consequently almost the whole VraSR regulon by CAMPs (III). In addition to vancomycin, VraSR is activated by other cell envelope interfering antimicrobial peptides, including bacitracin, mercacidin and daptomycin (Muthaiyan *et al.*, 2008; Sass *et al.*, 2008a; Utaida *et al.*, 2003). Surprisingly, the

results indicated that human cathelicidin LL-37 does not induce genes belonging to the VraSR regulon, the only exception being *prsA* (Table 7).

Table 7. Genes induced in S. aureus cells treated with LL-37

		Fold induction*			
Gene	**LL-37**	**Tem L**	**Ovi-1**	**Derm**	**Protein/Similarity**
clpB	2.2	3.9	4.1		ClpB chaperone homologue
ctsR	2.1	4.2	4.2		Transcription repressor of class III stress gene homologue
dnaJ	2.0	2.2	2.6		DnaJ protein
dnaK	2.1	2.5	2.9		DnaK protein
fhuG	2.0				Ferrichrome transport permease
groEL	2.3	3.8	4.3	2.2	GroEL protein
groES	2.0	3.2	3.6	2.4	GroES protein
lysC	2.0	5.8	8.3		Aspartokinase II
prsA	2.7	3.2	4.5	2.9	Peptidyl-prolyl cis/trans isomerase homologue
pyrE	2.1				Orotate phosphoribosyltransferase
pyrF	2.2				Orotidine-5-phosphate decarboxylase
ribA	2.1				Riboflavin biosynthesis protein
SA0205	2.1	17.2	11.2	6.2	Similar to lysostaphin precursor
SA0335	2.1	2.1	2.1		Component of twin-arginine translocation pathway
SA0336	2.2	2.2	2.1	2.1	Hypothetical protein
SA0481	2.0	4.1	4.0		Conserved hypothetical protein
SA0482	2.0	4.2	4.3		Putative ATP:guanido phosphotransferase
SA1406	2.0	2.4	2.9	2.1	Conserved hypothetical protein
SA1407	2.1	2.6	3.3		Conserved hypothetical protein
SA1599	2.1	3.2	2.4		Similar to transaldolase
SA1683	2.2				ABC transporter (ATP-binding protein) homologue
SA1820	2.1	3.6	3.0	2.2	Similar to bacteriophage terminase small subunit
SA1821	2.0	3.1	2.8	2.0	Hypothetical protein
SA2162	2.0	2.4	2.7		Similar to thioredoxin reductase
SAS016	2.5	5.0	7.4	5.4	Hypothetical protein
vraD	5.0	8.2	32.4	17.2	Similar to ABC transporter
vraE	2.0	3.4	11.8	6.2	Similar to ABC transporter

* For comparison, the induction ratios from DNA microarray experiments with other CAMPs are also listed if detected. tem L = temporin L, ovi-1= ovispirin-1, derm = dermaseptin K4-S4(1-16)

Strong induction of three operons emerged in all transcription profiles of CAMP-treated bacterial cells (III). These operons were *vraDE*, SA0205 and SAS016 encoding an ABC-type transporter similar to BceAB-like transporters in *B. subtilis* (Mascher *et al.*, 2003; Ohki *et al.*, 2003), a lysostaphin-like cell wall peptidase and a functionally unknown peptide of 55 amino acids, respectively. *vraDE* was also the most strongly induced operon in cells treated with LL-37 (Table 7). Surprisingly, no significant induction of the previously described genes contributing to CAMP resistance, such as *dlt* operon or *mprF* (Peschel *et al.*, 1999; Peschel *et al.*, 2001), was detected (III).

Antimicrobial peptide treatment also caused a response in general stress genes such as the *ctsR-clpC* operon, *groELS* and *dnaJK*, indicating that *S. aureus* cells try to adapt to the stress caused by CAMPs (III). Some of these target genes showed similar induction in *S. aureus* during phagocytosis by neutrophils or when surviving within epithelial or phagocytic cells (Garzoni *et al.*, 2007; Voyich *et al.*, 2005). Notably, several amino acid biosynthesis operons and genes encoding enzymes of the citric acid cycle were also induced (III). Simultaneous repression of genes

involved in anaerobic metabolism (Fuchs *et al.*, 2007) indicates that the cells were metabolically active and aerobically respiring. Another interesting phenomenon resulting from the CAMP treatments was the down-regulation of several virulence factors and their regulators, such as *saeRS* and *agr* (III).

5.2 ECF sigma factors and LiaRS-like TCSs mediates stress responses caused by damages in the cell envelope (I, III)

Transcription analysis revealed that CAMPs cause the induction of several signal transduction pathways. *In B. subtilis*, cell envelope stress caused by CAMPs was mainly mediated through ECF σ factors σ^W and σ^M and TCSs such as LiaRS (I). In *S. aureus*, strong activation of TCS VraSR was detected (III). All these regulatory systems have been reported to respond to disturbances of the cell envelope caused by a variety of cell envelope-interfering conditions.

5.2.1 SigW and SigM in *B. subtilis* (I)

A prominent finding in the transcriptome analysis was the activation of σ^W and σ^M ECF σ factors under CAMP stress (I, see also Tables 5 and 6 in section 5.1.1). σ^W also responds to a large number of antimicrobial compounds other than CAMPs, such as cephalosporin, vancomycin, D-cycloserine, cell membrane-active nigericin and Triton X-100, and more mildly to fosfomycin, bacitracin and tunicamycin (Cao *et al.*, 2002a). σ^M responds to several stress conditions interfering with the cell envelope, such as antibiotics, acid, ethanol, heat and superoxide stresses (Thackray & Moir, 2003).

The DNA macroarray results indicated that σ^W and σ^M have an important role in CAMP stress resistance in *B. subtilis*. However, neither σ^W, σ^M, nor the σ^W/σ^M double mutant showed increased sensitivity to any of the peptides used in this study (I). One possible explanation for this could be cross-regulation between different ECF σ factors. It has been demonstrated that regulons of different σ factors are partially overlapping, as in the case of σ^W, σ^M and σ^X (Cao *et al.*, 2002a; Huang *et al.*, 1998; Mascher *et al.*, 2007). Sigma factor σ^X also contributes to the maintenance of cell envelope integrity (Cao & Helmann, 2004), and it was induced by one peptide studied here, PG-1 (I). In order to find out more about the cross-regulation between different ECF σ factors, we carried out DNA array analysis with *sigM* and *sigW* knockout mutants using LL-37 for the induction. LL-37 treatment elicited a significantly lower number of induced genes in both σ mutants than in the wild type (I). As expected, the inactivation of one individual ECF σ factor silenced the genes it regulates, but the genes under the control of the other ECF σ factor were also induced at a decreased level. Surprisingly, the array data also showed that some genes that are expressed independently of σ^M and σ^W were also attenuated (I). These results suggest significant cross-talk between the σ^M and σ^W regulons, although it is apparent that not all the affected genes are directly regulated by σ factors. The functional overlap of several σ factors may be one possible explanation why the inactivation of σ^W, σ^M or both of them did not cause increased sensitivity to

antimicrobial peptides. This idea is further supported by the fact that the level of transcription of σ^W is elevated in cells lacking σ^X (Helmann, 2006; Huang *et al.*, 1998). The redundancy of σ^W, σ^M and σ^X regulons was confirmed in a recent study where inactivation of all three ECF σ factors made cells significantly more sensitive to several antibiotics compared to the wild type (Mascher *et al.*, 2007).

But what is the role of σ^W and σ^M in CAMP resistance, if any? Previously, it has been demonstrated that ECF σ factors are not active under normal laboratory growth conditions but are activated in specialized conditions such as cell envelope stress caused by cell envelope-interfering compounds (Helmann, 2002; Helmann, 2006). In fact, cells without any functional ECF σ factor present are still viable, at least under laboratory conditions (Asai *et al.*, 2008).

σ^W is the most thoroughly studied of the seven ECF σ factors found in *B. subtilis*. It is activated by several antibiotics and other cell envelope-interfering compounds. However, σ^W mutants only show increased sensitivity to fosfomycin (Cao *et al.*, 2002a). Thus, it seems that most of the σ^W-inducing agents are so-called gratuitous inducers: the activation of σ^W does not provide any advantage against the inducers (Helmann, 2006). This phenomenon is also seen in alkaline shock, where the σ^W regulon is strongly induced but inactivation of σ^W does not cause any significant changes in the sensitivity (Wiegert *et al.*, 2001). This might also be true in the case of CAMPs.

Recent studies have shed some light on the functional roles of the genes regulated by σ^W. Many of these genes were also induced by LL-37, PG-1 and/or PLL (Table 5). At least six operons controlled by σ^W have a verified role in resistance against antimicrobials, especially toxic peptides. SdpC is a toxic peptide produced by sporulating *B. subtilis* cells in order to lyse nonsporulating siblings in a process known as cannibalism (Ellermeier *et al.*, 2006; Gonzalez-Pastor *et al.*, 2003). Cells producing SdpC are resistant to its toxic effects, since they also simultaneously express immunity protein SdpI (Ellermeier *et al.*, 2006). In the absence of immunity protein SdpI, resistance to SdpC is mediated through σ^W. Two operons controlled by σ^W are associated with SdpC resistance: the *yknWXYZ* operon encoding ABC transporter and *yfhLM*, in which *yfhL* encodes a membrane protein homologous to SdpI immunity protein (Butcher & Helmann, 2006). Genes regulated by σ^W also contribute to resistance against the bacteriocin sublancin, which is encoded by the SPB prophage of *B. subtilis* (Butcher & Helmann, 2006).

B. subtilis strains are able to produce more than 20 antimicrobial compounds. The genes needed for the synthesis of toxic compounds as well as specific immunity genes are usually located on mobile genetic elements only present in some strains of *B. subtilis* (Stein, 2005). It has been proposed that σ^W provides broad-based intrinsic immunity to different kinds of antimicrobial compounds produced by *B. subtilis* or other closely related microbes (Helmann, 2006). From this perspective, it is not surprising that σ^W also responds to stress caused by CAMPs (I). CAMPs may cause similar disruption of the cell envelope than antimicrobials produced by bacterial

cells, leading to countermeasures by σ^W, even though they may not be efficient against CAMPs.

At the time the transcriptome analysis of *B. subtilis* cells treated with CAMPs was performed (I), little was known about the σ^M regulon and its function. Only a few genes had been verified to belong to the σ^M regulon (Horsburgh & Moir, 1999; Thackray & Moir, 2003). Today, knowledge of the σ^M regulon is more focused and ~57 genes in 30 operons have been identified to be regulated by σ^M (Eiamphungporn & Helmann, 2008; Jervis *et al.*, 2007). Many of these genes were previously assigned to σ^W and σ^X regulons. Due to the redundancy of antibiotic-inducible ECF σ sigma factors, σ^M does not usually cause increased sensitivity to cell envelope active compounds with the exception of bacitracin, moenomycin, SDS and some β-lactam antibiotics (Cao *et al.*, 2002b; Mascher *et al.*, 2007).

The σ^M regulon contributes to several central functions in the maintenance of cell envelope integrity, and its induction is referred to as a good reporter for interference in cell envelope synthesis and function (Eiamphungporn & Helmann, 2008). This is in agreement with our results (I), showing that CAMPs also activate many genes belonging to the σ^M regulon (Table 6). It has been demonstrated that σ^M regulates several other regulatory proteins, one of the most interesting being Spx, which controls several genes contributing to the maintenance of reducing conditions inside bacterial cells (Nakano *et al.*, 2005; Zuber, 2004). The gene encoding this transcription regulator, *spx* (formerly *yjbD*), was upregulated by all three CAMP treatments (I). It has been postulated that some bactericidal antibiotics generate reactive oxygen species (Kohanski *et al.*, 2007) and Spx-dependent genes may have a role in preventing cell death. Another interesting finding was the strong induction of *yrhH* by LL-37, and also to a lesser extent by PLL (I). *yrhH* encodes a putative methyltransferase and it has been suggested that the *yrhHIJ* operon might contribute to the regulation of membrane fluidity or in the defence against toxic fatty acids (Eiamphungporn & Helmann, 2008). The possible role of *yrhHIJ* genes in the CAMP resistance remains to be elucidated.

5.2.2 LiaRS and VraSR have different roles in the defence against antimicrobial agents (I, III)

LiaRS of *B. subtilis* and its homologue in *S. aureus*, VraSR, responded strongly to CAMPs (I, III). LiaRS (**l**ipid II-**i**nteracting **a**ntibiotics response **r**egulator and **s**ensor) controls the expression of *liaIH* genes with an unknown function and responds to several stress conditions. VraSR (**v**ancomycin **r**esistance **a**ssociated **s**ensor and **r**egulator) has been reported to react to several cell wall-interfering antibiotics. The genomic context of the *lia/vra* loci of *B. subtilis* and *S. aureus* differs from each other (Fig. 6), probably reflecting the differences in their physiological functions. The *liaIHGFSR* locus of *B. subtilis* contains six genes, while the *vra* locus of *S. aureus* contains only four genes. Homology can be found between the response regulators, sensor kinases and the inhibitory proteins (LiaF and SA1702), but not between the other proteins. The major difference between these two regulatory

systems seems to be the number of the genes they regulate. Only two operons, *liaIHGFSR* and *yhcYZ-yhdA*, are known to be under the regulation of LiaRS TCS, the *lia* operon being the main target (Jordan *et al.*, 2006). On the contrary, the large VraSR regulon in *S. aureus* comprises ~46 genes involved in cell wall associated functions such as protein quality control, protein folding and the modulation of cell wall biosynthesis (Kuroda *et al.*, 2003).

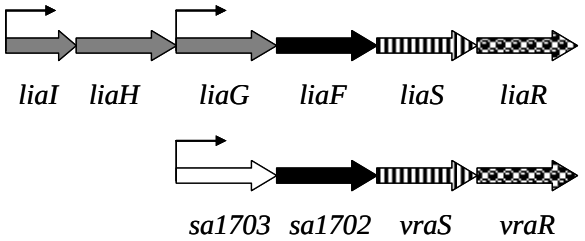

Figure 6. The genomic context of lia and vra operons. Genes are not drawn to scale. Dotted arrows represent response regulators and arrows with vertical lines represent histidine kinases. Gene encoding inhibitory protein LiaF and its homologue SA1702 are marked as black arrows. The functions of the other genes in the operons are unknown.

The *liaIH* genes were among the most strongly induced genes in *B. subtilis* cells treated with LL-37 (58-fold induction) and PG-1 (15-fold induction) (I). The *liaIH* genes reacted to CAMPs in a very rapid and transient way, as was shown in the qRT-PCR measurements; the expression of *liaI* was at the highest level after 10 minutes of exposure to LL-7 and declined to the basal level after two hours (I). The increase in *liaIH* expression was also confirmed at the level of translation, as LiaH was identified from the proteome analysis of cytoplasmic proteins (I). The function of LiaIH proteins is still a mystery. LiaI is a small hydrophobic protein with two putative transmembrane helices, while cytoplasmic LiaH shares some similarity with phage-shock protein PspA of *E. coli* possibly involved in the maintenance of cell envelope integrity (Darwin, 2005; Jordan *et al.*, 2007; Kleerebezem *et al.*, 1996). Even though the *lia* operon is strongly activated by cell wall-active antibiotics such as vancomycin, ramoplanin, bacitracin and nisin, the deletion of this operon did not cause increased sensitivity to any of the inducers tested (Jordan *et al.*, 2007; Mascher *et al.*, 2003; Mascher *et al.*, 2004). It is also activated to a lesser extent by various stress-causing compounds and conditions such as fosfomycin, tunicamycin, detergents, ethanol, phenol, alkaline shock and secretion stress, indicating that LiaRS TCS broadly senses various stress conditions (Hyyryläinen *et al.*, 2005; Mascher *et al.*, 2004; Petersohn *et al.*, 2001; Tam le *et al.*, 2006). In this study, both the natural CAMPs as well as Triton X-100 induced the *lia* operon, but somewhat surprisingly LiaRS did not respond to the synthetic peptide PLL (I).

The *lia* operon is also induced at the onset of the stationary phase without any exogenous stimuli (Jordan *et al.*, 2007). Cells entering the stationary phase have to adapt to the worsening living conditions, which may eventually lead to endospore formation (Errington, 2003; Msadek, 1999; Phillips & Strauch, 2002). The transition state regulator AbrB prevents P_{liaI} activity during logarithmic growth by directly

binding to the promoter region of *liaI*. In the stationary growth phase, AbrB repression is released by the Spo0A sporulation regulator and the *liaI* promoter is induced by some intrinsic stimulus (Jordan *et al.*, 2007). At least one intrinsic peptide encoded by *yydFGHIJ* operon activating LiaRS has been identified (Butcher *et al.*, 2007). Interestingly, a small peptide with 49 amino acids encoded by the *yydF* gene is predicted to be positively charged and able to adopt an α-helical conformation, both properties associated with CAMPs. It seems that *yydF* is a signalling molecule rather than an antimicrobial peptide (Butcher *et al.*, 2007).

Similarly to its homologue in *B. subtilis*, VraSR TCS in *S. aureus* was activated by the α-helical CAMPs temporin-L, dermaseptin and ovispirin-1 (III). The induction pattern of the VraSR-regulated genes was very similar to that observed with vancomycin-treated cells (Kuroda *et al.*, 2003). In contrast to LiaRS, the inactivation of *vraSR* leads to increased sensitivity to most of its inducers, as observed in previous studies with several cell wall-active antibiotics (Gardete *et al.*, 2006; Kuroda *et al.*, 2003). This was also seen in this study, as the *vraSR* deletion mutant was shown to be more sensitive to teicoplanin, bacitracin and a wide range of β-lactams, as well as to more unusual compounds with antimicrobial activity such as EGTA, phenothiazines and sodium tungstate (III). As CAMPs were strong inducers of the VraSR regulon, we expected to see increased sensitivity to CAMPs in *vraSR* deletion mutants. However, only slightly increased susceptibility to ovispirin-1 was observed. The inactivation of v*raSR* did not affect the sensitivity to other bacteriocins or CAMPs tested, including nisin, daptomycin, Pep5, LL-37 and hBD3 (III).

When the functional roles of the two homologous TCSs, LiaRS and VraSR, are compared, it seems that LiaRS is a general sensor for worsening living conditions and is activated by a broad spectrum of stress conditions, while VraSR protects the cells from various cell wall-disturbing agents. This is further supported by the fact that VraSR is activated by a variety of cell wall-active antibiotics, but is not induced by general stress conditions such as heat, osmotic shock or changes in pH (Kuroda *et al.*, 2003). The exact signal that these TCSs sense is still under investigation. It has been postulated that VraSR senses some byproduct formed when peptidoglycan sythesis is disturbed (Belcheva & Golemi-Kotra, 2008; Boyle-Vavra *et al.*, 2006). In support of this concept, VraSR is not activated in LL-37-treated cells (Table 7). The *S. aureus* strain Newman was found to be highly resistant to LL-37. Disturbances in the cell wall do not therefore probably take place and VraSR remains in inactivated stage.

5.3 BceRS-like TCSs specifically respond to antimicrobial peptides (I, III)

As LiaRS and VraSR seem to react to cell envelope stress in general, the specific recognition of toxic peptides is mediated through BceRS-like TCSs in both *B. subtilis* and in *S. aureus* (I, III). Characteristic of TCSs of this kind is that they control the expression of the ABC transporter encoding genes adjacent to them. The genome of *B. subtilis* harbors three TCS-ABC transporter systems of this kind: *yxdJK-yxdLM*, *bseRS-bceAB* and *yvcPQ-yvcRS* (Joseph *et al.*, 2002). The *yxdLM* operon was strongly and specifically induced by LL-37 (I). Two homologues of *yxdLM* can be found from the *S. aureus* genome: *vraDE* and *vraFG*. The former was strongly activated by all CAMPs tested, while only slight induction of *vraFG* was detected in the qRT-PCR measurements (III). The genomic context of these two ABC transporter operons of *S. aureus* differs somewhat from that found in *B. subtilis*. An operon encoding a three-component regulatory system known as Aps (also referred to as GraRS) (Li *et al.*, 2007a; Meehl *et al.*, 2007) is located adjacent to *vraFG* operon, whereas no other operon can be identified from the immediate proximity of *vraD* (Fig. 7).

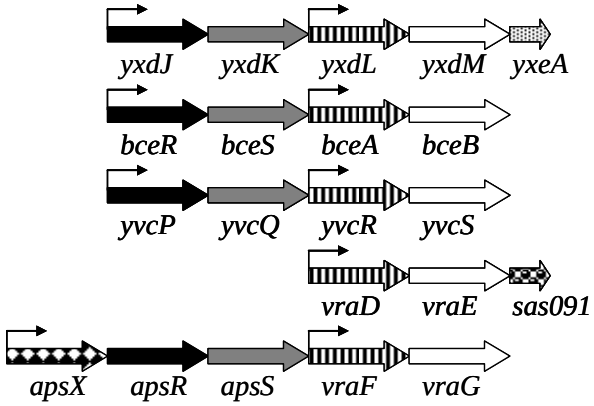

Figure 7. Comparison of the genomic context of yxdJK-yxdLM operons and their homologues in B. subtilis and S. aureus. Genes are not drawn to scale. Black arrows represent response regulators and grey arrows sensor kinases of two-component systems. Arrows with vertical lines represent the ATP-binding proteins and white arrows the permease proteins of ABC transporters. The function of ApsX, YxeA and SAS091 is unknown.

5.3.1 LL-37 is a specific inducer of the YxdLM ABC transporter (I)

LL-37 was revealed to be a strong and specific inducer of the *yxdLM* operon in *B. subtilis* (I). Both *yxdLM* homologues, *bceAB* and *yvcRS*, were also slightly induced in cells treated with LL-37, but clearly to a lesser extent compared to *yxdLM* (I). Transription of *yxdLM, bceAB* and *yvcRS* operons is controlled by the adjacent TCS responding to the extracellular presence of antimicrobial compounds (Joseph *et al.*, 2002; Joseph *et al.*, 2004; Mascher *et al.*, 2003). The regulation of these three operons was further studied by determining their expression in the wild-type strain and mutants of the upstream histidine kinase genes (*yxdK*, *yvcQ* and *bceS*) treated with LL-37 by using qRT-PCR (I, Table 8). The results suggest that the expression of *yxdLM* is tightly regulated by YxdJK and not affected by the two other sensory systems (I). The slight downregulation of *yxdL* gene observed in the *yvcQ* and *bceS* mutants probably resulted from the high variation between individual qRT-PCR measurements. On the contrary, it seems probable that expression of the *yvcRS* operon is partly regulated by YxdJK, whereas expression of the *bceAB* operon is not affected either by YxdJK or YvcQP (I). The cross-regulation between these TCS ABC transporter systems is further supported by a recent study in which it was demonstrated that the YvcPQ-YvcRS system indirectly responds to bacitracin only at high concentrations in a BceRs-BceAB-dependent manner (Rietkötter *et al.*, 2008).

Table 8. Cross regulation of the BceRS-like TCSs

TCS-mutation	**Transcription of operon**		
	yxdLM	*yvcPQ*	*bceAB*
yxdJK	0	↓	no effect
yvcQP	(↓)	↓	no effect
bceSR	(↓)	no effect	↓

The effect of inactivation of TCS on the transcription of ABC transporter encoding genes: 0 no transcription, ↓ decreased transcription, no effect = transcription level same as in WT, parentheses = decreased transcription is probably due to the high variation between separate experiments

Studies with *B. subtilis* have revealed that ABC transporters encoded by *yxdLM* and its homologues have a role in the removal of harmful constituents. The BceRS-BceAB system is the best characterized of these detoxification modules and it has been shown to be the primary and most efficient factor in bacitracin resistance (Joseph *et al.*, 2002; Joseph *et al.*, 2004; Mascher *et al.*, 2003; Ohki *et al.*, 2003). Bacitracin is a cyclic nonribosomally synthesized dodecylpeptide produced by *B. licheniformis* and some *B. subtilis* strains (Azevedo *et al.*, 1993; Ishihara *et al.*, 2002). The direction of bacitracin transport by BceAB is still a matter of debate (Bernard *et al.*, 2007; Rietkötter *et al.*, 2008). The YvcPQ-yvcRS system is also

induced by bacitracin and nisin, but its exact function is still unclear (Jordan *et al.*, 2008; Mascher *et al.*, 2003).

Despite the strong induction of the *yxdLM* operon by LL-37, it seems that LL-37 is not the substrate for the YxdLM ABC transporter. Neither the *yxdL* mutant nor the *yxdL/yvcR* double mutant showed increased sensitivity to LL-37 (I). It is possible that LL-37 resembles some as yet unidentified molecule and is therefore able to activate the YxdJK sensory system. The specific activation of YxdJK TCS only by LL-37 may indicate direct binding of activating ligand to the sensor kinase. Direct sensing of a signal molecule has been postulated for the BceRS-BceAB system (Rietkötter *et al.*, 2008). Interestingly, it seems that bacitracin is sensed during its transport through BceAB, and the large periplasmic loop of the BceA permease component of the transporter may be involved in mediating the signal to the BceRS TCS (Rietkötter *et al.*, 2008). The function of YxdJK-YxdLM remains to be elucidated, but it is still tempting to speculate that it plays some role in resistance against toxic peptides.

5.3.2 VraDE confers resistance to bacitracin (III)

All CAMP treatments caused strong induction of the ABC transporter encoding the *vraDE* operon in *S. aureus*. In addition to CAMPs, bacitracin also caused strong, almost 500-fold induction of gene expression (III). VraDE induction has also been demonstrated with other antimicrobial peptides such as human β-defensins and the lantibiotic mersacidin (Sass *et al.*, 2008a; Sass *et al.*, 2008b). In order to determine whether *vraDE* has any role in resistance against antimicrobial compounds, susceptibility tests with *vraDE* mutants were performed (III). The CAMPs tested included ovispirin-1-NH_2, temporin L-NH_2, two lantibiotics (nisin and Pep5), human cathelicidin LL-37, and hBD3 defensin. In addition, MICs for vancomycin, teicoplanin, bacitracin and daptomycin were determined. In contrast to the results of a previous study postulating the possible role of VraDE in CAMP resistance (Sass *et al.*, 2008b), the *vraDE* mutant showed increased sensitivity to bacitracin (about 10-fold difference in MIC), but no other differences were observed in antimicrobial sensitivities (III). These results strongly indicate that VraDE is a bacitracin-specific detoxification module, similarly to BceAB in *B. subtilis*.

As mentioned above, no TCS-encoding operon is located adjacent to *vraDE* genes (Fig. 7). Expression of the *vraDE* operon is controlled by the Aps (also referred to as GraRS) system. Aps also regulates the expression of the neighbouring *vraFG* operon encoding an ABC transporter homologous to *vraDE*. In addition, Aps controls the expression of *mprF* and *dlt* operons responsible for the overall charge of the cell envelope (Kraus *et al.*, 2008; Li *et al.*, 2007a). It seems that Aps is one of the major mediators in CAMP-induced stress responses in both *S. aureus* and *S. epidermidis* (Li *et al.*, 2007a; Li *et al.*, 2007b). This is further supported by the observation that natural mutation in the ApsS sensor of the *S. aureus* strain SG511 leads to increased susceptibility to CAMPs (Sass & Bierbaum, 2009). The structure of the sensor component ApsS of *S. aureus* differs from that of *S. epidermidis*, leading to the differential CAMP recognition in these two bacterial species. Furthermore, it seems that the short extracellular loop of ApsS directly interacts with CAMPs, activating

the sensor (Li *et al.*, 2007a). Our results also indicated that the C-terminal amide group in linear peptides is an important element for the activation of the Aps sensory system, as high-level induction of *vraDE* by ovispirin-1 was shown to be dependent on the amide modification of the C-terminus (III).

It has also been demonstrated that the VraFG transporter confers resistance to several CAMPs, including hBD3, nisin, indolicidin and LL-37 (Li *et al.*, 2007a). It is, however, quite surprising that only slight induction of the *vraF* gene by ovispirin-1-NH2 and no induction at all by other peptides was detected (III). Considering the simultaneous strong induction of *vraDE*, it is obvious that other regulatory elements than Aps must also be involved in the regulation of *vraDE* expression (III). The existence of an unidentified regulator of *vraDE* was also suggested in a recent study by Li *et al*. (2007a).

5.4 D-alanylation of teichoic acids affects the signal sensing by TCSs (II)

The Dlt system seems to play a crucial role in resistance against CAMPs in *S. aureus* and other Gram-positive bacteria (Fabretti *et al.*, 2006; Kovacs *et al.*, 2006; Peschel *et al.*, 1999). In *B. subtilis*, DltA, DltB, DltC and DltD comprise the Dlt system responsible for the D-alanylation of both lipoteichoic and wall teichoic acids, leading to a decreased net negative charge in the cell wall matrix (Perego *et al.*, 1995). In this study we wanted to investigate how the charge of the cell envelope affects signal sensing through TCSs. The TCSs of interest were CssRS, LiaRS and YxdJK. CssRS was initially found out to participate in the maintenance of cell envelope integrity under secretion stress. It responds to the accumulation of misfolded proteins and controls the expression of *htrA* and *htrB* genes encoding serine-type surface proteases involved in quality control (Darmon *et al.*, 2002; Hyyryläinen *et al.*, 2001). Interestingly, inactivation of either CssS or DltD seems to cause similar effects on the quality control of secreted proteins, as the stability and secretion of some misfolded or heterologous proteins is improved in both of the mutants (Hyyryläinen *et al.*, 2000; Hyyryläinen *et al.*, 2001; Vitikainen *et al.*, 2005).

The TCSs of interest were induced either by secretion stress caused by α-amylase (AmyQ) hypersecretion from the plasmid pKTH10 or by treating cell cultures with LL-37 or other cell envelope-interfering substances. The effects of inactivation of the Dlt system on the activity of three different TCSs of *B. subtilis* were analyzed by β-galactosidase assays and qRT-PCR measurements.

In order to determine how the *dlt* mutation affects the expression of *htrA* and *htrB* genes, a *lacZ* reporter gene was inserted into *htrA* and *htrB* genes, enabling the measurement of transcription activity from the promoters P_{htrA} and P_{htrB} using β-galactosidase assays. AmyQ hypersecretion resulted in an approximately 10-fold induction of expression in the wild type strain, and similar induction of expression from the P_{htrB} promoter was also detected (II). On the contrary, hardly any induction of P_{htrA} and P_{htrB} was observed in the *dltD* mutant (II). These results were also

verified by qRT-PCR measurements suggesting that the D-alanylation of teichoic acids modulates signal transduction via CssRS. We were also able to demonstrate a decreased level of HtrA protein in the culture medium of the *dlt* mutant compared to the wild type (II).

Another TCS responding to severe secretion stress in *B. subtilis* is LiaRS (Hyyryläinen *et al.*, 2005). Interestingly, the effect of the *dlt* inactivation was quite opposite to that seen in the case of CssRS. LiaRS was activated about five-fold higher in the *dlt* mutant than in the wild type strain under secretion stress (II). When LL-37 was used as an inducer of LiaRS, the increased activity of LiaRS in the *dlt* mutant was even more clearly seen. The enhanced induction was observed with both a P_{liaI}–*lacZ* reporter and by measuring P_{liaI} expression with qRT-PCR. YxdJK was the third sensory system used to study the Dlt dependence of TCS induction. Similarly to LiaRS, YxdJK activity was enhanced in the *dlt* mutant (II).

Whether the density of the negative charge in the cell wall also modulates the response of LiaRS to other cell envelope-interfering compounds with non-ionic nature was also determined. For this purpose, *B. subtilis* cells were treated with the detergent Triton X-100 or vancomycin. Vancomycin is known to be a strong inducer of LiaRS (Mascher *et al.*, 2004), and the activity of LiaRS was enhanced hundreds-fold in the wild-type strain compared to the expression level in non-treated cells (II). Interestingly, the response to vancomycin was significantly lower in the *dltD* mutant in contrast to the results obtained from the experiments with LL-37. Triton X-100 caused similar induction of LiaRS in both the wild type strain and *dlt* mutant (II).

The effect of the Dlt system on the induction patterns of CssRS, LiaRS and YxdJK may indicate that *B. subtilis* can modulate cell signalling via TCSs by controlling the density of the negative charge of the cell envelope, but other possible explanations also exist. The decreased activity of CssRS in the *dlt* mutant may result from improved post-translocational protein folding due to the binding of divalent cations to the cell wall with an increased net negative charge (Hyyryläinen *et al.*, 2000). Similarly, the increased negative charge of the cell wall in cells lacking the Dlt system facilitates the access of cationic peptides to the cell surface (Peschel *et al.*, 1999), which might explain the enhanced activity of LiaRS and YxdJK in LL-37-treated cells. This is further supported by the observation that non-ionic Triton X-100 did not cause differential activity of LiaRS in wild type or *dlt*-mutant strains (II). The decreased expression of genes regulated by LiaRS in the vancomycin-treated cells probably reflects the different action mechanims of CAMPs and vancomycin. The altered charge of the cell wall due to *dlt* mutation probably does not affect the action of uncharged vancomycin. It is still puzzling why the activity of LiaRS was decreased in the *dlt* mutant compared to the wild type after vancomycin treatment. The glycopeptide antibiotic vancomycin inhibits PG synthesis by binding to the lipid II precursor, but in addition it can bind to free D-ala-D-ala termini present in the cell wall (Daniel & Errington, 2003; Sheldrick *et al.*, 1978). As mentioned before, LiaRS does not sense vancomycin directly, but rather the disturbances it causes in the cell envelope. However, it is possible that when D-alanyl esters are absent from teichoic acids, the binding of vancomycin to the cell wall is diminished and the overall cell envelope stress is alleviated.

The importance of the cell wall in protection against CAMPs is emphasized by the observation that the charge of the cell membrane did not affect to the signal sensing through TCSs of interest (II). This was demonstrated by two mutant strains with an altered membrane charge. The inactivated components were MprF, which modifies membrane lipids with L-lysine (Peschel & Collins, 2001; Staubitz & Peschel, 2002), and *psd*, which encodes phosphatidylserine decarboxylase and is involved in the biosynthesis of phosphatidylethanolamine (Cao & Helmann, 2004; Matsumoto *et al.*, 1998). Both of these mutations lead to an increased negative charge of the cell membrane. Compared to the wild type, the mutations had no significant effects on the activity of LiaRS or YxdJK after LL-37 treatment. These results suggest that the membrane surface charge does not modulate signalling via the TCSs (II).

5.5 Is cell wall synthesis also a target for CAMPs? (I, III)

One of the purposes of this study was to determine whether transcriptome profiles could tell something about the mechanims of action of different types of CAMPs. The overall impression from DNA array experiments was that all CAMPs studied here appeared to elicit similar kinds of stress responses in bacterial cells, with only a few undisputable exceptions such as the *yxdLM* operon in *B. subtilis* (I, III). As membrane disruption is considered to be the primary mechanism of the CAMP-mediated killing of bacterial cells, the changes in gene expression were surprisingly similar to those seen in the transcriptome analysis with cell wall-interfering antibiotics such as vancomycin (Cao *et al.*, 2002b; Kuroda *et al.*, 2003; Utaida *et al.*, 2003). This naturally raises the question of whether cell wall synthesis is an additional target for CAMPs. In addition to the effects on membranes, several lantibiotics such as nisin and mersacidin produced by Gram-positive bacteria inhibit cell wall biosynthesis (Breukink *et al.*, 1999; Brotz *et al.*, 1998a; Brotz *et al.*, 1998b). The target of both peptides is Lipid II, the basic peptidoglycan building block. Nisin uses Lipid II as a docking molecule to form pores in the membrane, whereas mersacidin inhibits the transglycosylation reaction of cell wall biosynthesis (Breukink & de Kruijff, 2006; Brotz *et al.*, 1997). On the contrary, specific receptors for CAMPs of animal origin have rarely been described (Yeaman & Yount, 2003). Thus, instead of binding to a specific target, it seems more likely that the possible cell wall defect caused by CAMPs results from the unspecific binding of positively charged peptides to the negatively charged components of the cell wall. Another possible explanation could be that CAMPs reactivate otherwise inert autolysins attached to the peptidoglycan, leading to increased cell wall turnover, as has been reported in the case of the lantibiotics nisin and Pep5 (Bierbaum & Sahl, 1985; Bierbaum & Sahl, 1987). One of the most strongly induced genes in CAMP-treated *S. aureus* cells was SA0205, encoding a lysostaphin-like cell wall peptidase (III). On the other hand, it should be remembered that most of the components of the cell wall are synthesised by integral membrane proteins. Thus, even if the main target of CAMPs is the cell membrane, as generally suggested, it seems reasonable that severe membrane disruption caused by CAMPs also reflects cell wall biosynthesis. The possible role of CAMPs in causing defects in the cell wall must be verified in future studies.

5.6 General challenges in transcriptome analysis of bacterial cells (I,III)

The DNA arrays used in this study are powerful tools in providing insights into the changes in gene expression of bacterial cells under varying living conditions. They may also help to characterise the possible roles of the genes of unknown function. The great advantage of the DNA array technique is that it enables the analysis of the whole genome at the same time. Nonetheless, there are notable challenges when the DNA array technique is used to identify genes responding to a particular stress condition, and especially in defining the role of different regulatory elements in the response. The genes are often controlled by multiple regulatory proteins and the contribution of one regulator can be easily masked by other regulators. In addition, it is difficult to distinguish the indirect effects caused by the activation of a certain regulatory element (Helmann, 2006). It is also debated that the sublethal concentrations of antimicrobial peptides used in many transcriptome analysis represent a sudden antibiotic shock rarely reflecting the natural situation, at least in the case of *B. subtilis* (Rietkötter *et al.*, 2008).

In this study, a two-fold change in gene expression was considered as a significant change (I,III). However, it is obvious that the collected data included several genes that were false positives or had no significant role in the stress response. On the other hand, some relevant genes may have been discarded from the final results. The outcome of the analysis could be improved by using more biological replicates and more accurate statistical methods. The high variation between experiments, especially in the case of the highly induced genes, complicated the analysis of results. This was particularly problematic in the data analysis of the qRT-PCR results. The changes in expression of the induced genes were clearly higher in the qRT-PCR measurements than in the DNA microarray, but overall consistency was found in the results (I, III). It should also be remembered that the high induction of a particular gene does not automatically mean that the gene in question has a direct role in stress tolerance. This was seen, for example, in the case of the LiaRS system in *B. subtilis* (I).

In addition, the possible differences in culture conditions, sample handling and in performing the DNA array or qRT-PCR experiments may have caused variation in the results. One of the most important factors affecting the final results was the quantity of peptides used in the experiments, and especially the definition of the sublethal concentrations for each peptide. The antibacterial activity of a particular peptide may vary depending on the culture medium used in experiments, as well as the peptide batch in use. In addition, the possible proteolytic degradation of peptides during storage or sample preparation could not been monitored. To avoid these problems, all the samples compared to each other within an experiment were simultaneously cultured and similarly handled.

6 Conclusions

The bacterial responses to stress caused by cationic antimicrobial peptides were investigated in the present study. Two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, were selected for transcriptome analyses examining the changes in transcription under peptide stress. This study contributes to the understanding of how bacteria can resist and adapt to the effects of toxic peptides, which may give new insights into future drug development.

Two main mechanisms mediating the stress responses were revealed in the transcriptome analyses: ECF σ factors and two-component systems (TCSs). In *B. subtilis*, σ^W, σ^M and LiaRS were demonstrated to play an important role in sensing general stress in the cell envelope caused by CAMPs. As the *S. aureus* genome harbors only one putative ECF σ factor, TCSs seem to be the major sensor system in CAMP stress in *S. aureus*. VraSR, homologous to LiaRS of *B. subtilis*, has previously been associated with resistance to antibiotics that interfere with the cell wall, and it was also strongly activated by CAMPs in the present study. However, inactivation of any of the regulatory systems mentioned above did not cause significantly increased sensitivity to CAMPs. In the case of ECF σ factors, one reason for this might be the redundancy of different ECF σ regulons. Significant cross-regulation between σ^W and σ^M was also demonstrated in our study. LiaRS seems to be a general sensor for worsening living conditions and might have a role in the process leading to spore formation rather than directly in stress tolerance. VraSR does not respond to general stress conditions, but instead confers resistance to vancomycin and other glycopeptide antibiotics. It propably senses some byproduct resulting from disturbance in the cell wall, indicating that CAMPs might also interfere in cell wall assembly.

The BceRS-like TCSs seem to be responsible for the direct recognition of antimicrobial peptides. Typical for these TCSs is that they regulate the expression of adjacent genes encoding ABC transporters such as YxdLM in *B. subtilis*. *yxdLM* and its homologue *vraDE* in *S. aureus* were the most highly induced operons in the transcriptome analyses. *yxdLM* was only induced by human cathelicidin LL-37, whereas *vraDE* was strongly activated by all CAMPs tested and also by bacitracin. The exact role of YxdLM remains to be elucidated, as it seemed likely that LL-37 is not the substrate for it. Our results strongly suggest that the VraDE ABC transporter confers resistance to bacitracin, like its homologue BceAB in *B. subtilis*.

Transcriptome analysis did not reveal any specific extrusion or inactivation mechanism against CAMPs of animal or human origin in either *B. subtilis* or *S. aureus*. It seems that, for example, BceAB-like transporters have primarily evolved to serve in the active extrusion of antimicrobial peptides produced by competiting bacterial species living in the same habitat. In addition, bacterial species producing toxic peptides themselves have to have mechanisms against the harmful effects of

the produced peptide, as is proposed for the function of σ^W in *B. subtilis* (Helmann, 2006).

An increase in the net positive charge of the cell wall is proposed to be the main defence mechanism of bacterial cells against CAMPs. This is mainly achieved by the Dlt system responsible for the D-alanylation of teichoic acids, leading to an increased positive charge of the cell wall (Perego *et al.*, 1995; Peschel *et al.*, 1999). In the research for this thesis, we also demonstrated that the regulation of transcription by TCSs is influenced by the Dlt system in *B. subtilis*. Inactivation of the Dlt system differentially affects the activity of different TCSs, depending on their functional role in the cells and the stimuli they sense. The activity of LiaRS and YxdJK was increased in *dlt* mutants under CAMP stress, indicating that access of CAMPs to the cell membrane was enhanced. Interestingly, the alterations in the charge of the cell membrane did not cause differential transcription via LiaRS or YxdJK. This observation further supports the importance of the cell wall in protecting bacterial cells from positively charged toxic peptides.

Taken together, CAMPs elicit complex stress responses in both *B. subtilis* and *S. aureus*, involving several sensory systems and also some as yet unidentified regulatory elements. This does not mean that all the signalling pathways activated in CAMP stress specifically act in CAMP defence, but rather that they respond to the diversity of environmental changes depending on the lifestyle of the particular bacterial species.

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Milla Pietiäinen

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Original Publications

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Cationic antimicrobial peptides elicit a complex stress response in *Bacillus subtilis* that involves ECF-type sigma factors and two-component signal transduction systems

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Stress responses of *Bacillus subtilis* to membrane-active cationic antimicrobial peptides were studied. Global analysis of gene expression by DNA macroarray showed that peptides at a subinhibitory concentration activated numerous genes. A prominent pattern was the activation of two extracytoplasmic function sigma factor regulons, SigW and SigM. Two natural antimicrobial peptides, LL-37 and PG-1, were weak activators of SigW regulon genes, whereas their synthetic analogue poly-L-lysine was clearly a stronger activator of SigW. It was demonstrated for the first time that LL-37 is a strong and specific activator of the YxdJK two-component systems, one of the three highly homologous two-component systems sensing antimicrobial compounds. YxdJK regulates the expression of the YxdLM ABC transporter. The LiaRS (YvqCE) TCS was also strongly activated by LL-37, but its activation is not LL-37 specific, as was demonstrated by its activation with PG-1 and Triton X-100. Other strongly LL-37-induced genes included *yrhH* and *yhcGHI*. Taken together, the responses to cationic antimicrobial peptides revealed highly complex regulatory patterns and induction of several signal transduction pathways. The results suggest significant overlap between different stress regulons and interdependence of signal transduction pathways mediating stress responses.

INTRODUCTION

Our purpose in this study was to characterize stress responses of *Bacillus subtilis* to cationic antimicrobial peptides. Antimicrobial peptides represent an ancient form of weapon in host defence mechanisms and their ubiquitous existence in cells and organisms of all types suggests important roles for them in innate immunity and defence against microbial invasion (reviewed by, for example, Yeaman & Yount, 2003). Natural antimicrobial peptides are typically amphipathic and positively charged, and they contain well-defined α-helical or β-sheet structures (see also below). Mammalian antimicrobial peptides include defensins, protegrins and cathelicidins. Antimicrobial peptides typically attach to membrane surfaces of invading pathogens and disturb membrane integrity (Zasloff, 2002). In the case of nisin and epidermidin, however, a specific target has been identified. They interact with the membrane-bound peptidoglycan precursors and disturb cell wall biosynthesis (Breukink *et al.*, 1999; Brotz *et al.*, 1998). Modification of the net negative charge of the bacterial cell surface by adding positively charged residues to teichoic and lipoteichoic acids helps bacteria to avoid being killed by antimicrobial peptides. It has been shown that inactivation of the *dlt* operon of *Staphylococcus aureus* and consequent lack of D-alanine substitution in teichoic and lipoteichoic acids results in increased negative charge of the cell surface and increased sensitivity to defensins, protegrins and other antimicrobial peptides (Peschel *et al.*, 1999). The absence of D-alanine substitution in the anionic polymers of *Listeria monocytogenes* also increases sensitivity to antimicrobial peptides and decreases virulence (Abachin *et al.*, 2002).

Extracytoplasmic function (ECF) sigma factors are regulatory components by which bacteria control gene expression in response to environmental stress. There are seven different ECF-type sigma factors in the Gram-positive model bacterium *B. subtilis* (Helmann, 2002; Kunst *et al.*, 1997). Several stress conditions activate the SigW regulon. These include alkaline shock (Wiegert *et al.*, 2001), inhibition of the cell wall synthesis by antibiotics such as vancomycin and disturbance of the integrity of the cell membrane by detergents (Cao *et al.*, 2002b). The SigW sigma factor is associated with the membrane-bound SigW anti-sigma factor when the *Bacillus* cell is not exposed to environmental

Abbreviations: ECF, extracytoplasmic function; PLL, poly-L-lysine; TCS, two-component system.

stress (Schobel *et al.*, 2004). Under stress conditions, the anti-sigma factor is proteolytically degraded, resulting in the release of SigW from the membrane and binding to gene promoters of the regulon (Schobel *et al.*, 2004). A similar pattern is anticipated for other ECF-type sigma factors. SigM is required for combating stress due to antibiotic effects on the cell wall, ethanol, heat, acid and superoxide (Thackray & Moir, 2003). It is also essential for survival in environments containing high concentrations of salt (Horsburgh & Moir, 1999), suggesting that it is required for maintaining the integrity of the cell envelope. Alternative sigma factors are not the only regulatory systems that are involved in stress tolerance: two-component systems (TCSs) also have a role in controlling gene expression in environmental changes. TCSs are signalling devices composed of a membrane-bound sensor kinase and a response regulator. *B. subtilis* two-component regulation has recently been reviewed (Ogura & Tanaka, 2002).

We studied stress responses to two naturally occurring antimicrobial peptides, LL-37 and PG-1, and their synthetic analogue poly-L-lysine (PLL), using a DNA macroarray and real-time RT-PCR. Human LL-37 is 37 amino acid residues long and belongs to the cathelicidin family of antimicrobial peptides (Johansson *et al.*, 1998; Turner *et al.*, 1998). It is an amphipathic and α-helical peptide that probably disrupts the lipid bilayer by a toroidal pore mechanism (Henzler Wildman *et al.*, 2003; Johansson *et al.*, 1998). The porcine protegrin PG-1 is composed of 18 amino acid residues, including four cysteines, and forms a two-stranded antiparallel β-sheet linked by a β-turn (Aumelas *et al.*, 1996; Fahrner *et al.*, 1996). The four cysteines of PG-1 form two disulphide bonds, which are important for the β-sheet conformation and antimicrobial activity (Harwig *et al.*, 1996). The synthetic peptide PLL differs from the natural peptides in that it is not amphipathic. Its mode of action on membranes is unclear.

It was found that the antimicrobial peptides induced ECF-type sigma factor regulons in a complex manner. Several genes that are regulated by two-component signal transduction systems were also induced. Most interestingly, LL-37 strongly upregulated, via the YxdJK TCS, the *yxdLM* genes encoding an ABC-type transporter of unknown function. The *yvcRS* and *bceAB* (*ytsCD*) genes, which encode ABC transporters highly homologous with YxdLM, were also moderately upregulated. Interestingly PG-1, PLL and Triton X-100 did not induce the expression of any of these ABC-transporter genes.

METHODS

Bacterial growth conditions. *B. subtilis* cells were grown in Luria–Bertani (LB) medium or in BFA minimal medium at 37 °C with vigorous shaking. The BFA medium is a modified Spizizen's minimal salts medium (Anagnostopoulos & Spizizen, 1961) containing glutamine instead of ammonium sulphate as the nitrogen source. When needed, kanamycin and erythromycin were added at concentrations of 10 μg ml^{-1} and 1 μg ml^{-1}, respectively. *B. subtilis* strain 168 and its derivatives were used in all experiments. The sensitivity of strains to four antimicrobial peptides, HNP-1 (American Peptide Company), PG-1 (Bachem), LL-37 (Ale Närvänen, University of Kuopio, Finland) and PLL (Sigma-Aldrich) were tested with the Bioscreen C Microbiology Reader (Labsystems). The culture volume was 150 μl and the number of bacteria in the inoculum was approximately 10^6 ml^{-1}. These tests were also repeated in shake-flask cultures at approximately 10^8 bacteria (ml inoculum)$^{-1}$.

Mutant constructions. The *sigW*::*neo* and *sigM*::pMUTIN4 mutations were introduced into strains by transformation with chromosomal DNA of the *B. subtilis* strains HB4247 (kindly supplied by J. D. Helmann) and MJH003 (Horsburgh & Moir, 1999), respectively. The *sigW sigM* double mutant was constructed by transforming the strain IH8342 containing the *sigW*::*neo* mutation with the chromosome of MJH003. The *yvcQ* gene was inactivated with pMUTIN4, as described by Vagner *et al.* (1998). The *yxdJ* and *yxdK* null mutations were obtained from Naotake Ogasawara (Nara Institute of Science and Technology, Nara, Japan).

RNA isolation, labelling with ^{33}P and DNA macroarray analysis. For RNA isolations, strains were grown in the BFA minimal medium containing 100 mM NaCl in shake-flask cultures. Cell densities were measured with a Klett colorimeter. Antimicrobial peptides were added at 60 Klett units and samples for RNA isolation were harvested after 20 min from 4 ml cell culture by centrifugation. Control samples without peptides were treated in a similar manner. Cells were resuspended in 400 μl ice-cold culture medium and transferred to screw-capped Eppendorf tubes containing 1·5 g glass beads, 50 μl 10 % SDS, 50 μl 3 M sodium acetate and 500 μl phenol/chloroform/isoamylalcohol (25 : 24 : 1 by vol.). The tubes were frozen in liquid nitrogen, followed by vigorous shaking for 6 min with a face-grinding machine and centrifugation at 10 000 r.p.m. for 5 min. The water phase was mixed (Vortex) with 1 vol. chloroform and centrifuged at 14 000 r.p.m. for 2 min. Next, the water phase was mixed with 2 vols Roche lysis/binding buffer, and the RNA extraction was continued with the Roche High Pure RNA Isolation Kit according to the manufacturer's instructions.

DNA macroarray analysis was carried out using Panorama *B. subtilis* gene array filters and specific cDNA labelling primers (Sigma Genosys). The Panorama *B. subtilis* gene array contains duplicate spots of PCR products representing currently known *B. subtilis* genes. Prior to cDNA synthesis, the quality of RNA was confirmed using Northern blotting. For cDNA synthesis, 10 μg RNA was used, and the synthesis was performed as described by Wiegert *et al.* (2001). The SuperScript II reverse transcriptase was purchased from Gibco-BRL. cDNA was purified with MicroSpin G-25 columns (Amersham Pharmacia Biotech) and the labelling efficiency was determined with a liquid scintillation counter. Prehybridization, hybridization and washing of the filters were performed according to the manufacturer's instructions. The DNA array filters were exposed overnight on phosphor screens and the screens were scanned with a Fluorescent Image Analyser FLA-2000 (Fujifilm). Hybridization signal intensities were quantified with the ArrayVision software (Imaging Research), as described by Wiegert *et al.* (2001). Data were filtered to avoid false positives by excluding genes with a signal-to-noise ratio <3 (Array Vision software) and normalized by dividing the intensity of each spot by the mean intensity of all the spots. Each experiment was carried out twice with RNA isolated from two independent cultures. Genes were regarded as induced when the induction ratio was >2 in both experiments.

Quantitative real-time RT-PCR. For real-time RT-PCR, RNA was isolated similarly as for the DNA array. RT reactions were carried out with the Omniscript Reverse Transcriptase Kit (Qiagen) according to the manufacturer's instructions with the exception of an additional DNase I (Roche) treatment. An equal amount of RNA (2 μg)

was used in each RT reaction. Primers used in RT reactions were random hexamers (0·15 μg ml^{-1}) provided by Roche. The absence of chromosomal DNA in the RNA preparations was verified with a control sample that was not treated with RT, but was otherwise treated in a similar manner to the RT-treated samples. Real-time PCR reactions were carried out with specific primer pairs using the SYBR green PCR master mix (Applied Biosystems). Primers were designed with the Primer Express software (Applied Biosystems) and purchased from Sigma Genosys or TAGC Copenhagen. Sequences of the PCR primers for the genes studied are shown in Table 1. The amplification and detection of PCR products were performed with the ABI PRISM 5700 sequence detection system (Applied Biosystems). The cycling conditions were: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold cycle (Ct) is the first cycle at which the fluorescence becomes detectable above the background and is inversely proportional to the logarithm of the initial number of template molecules. Ct values of known quantities of *B. subtilis* chromosomal DNA were plotted for each primer pair to obtain standard curves. The standard curves allowed us to convert the Ct values of each amplified gene in the cDNA preparations to relative numbers of cDNA molecules. These cDNA values were normalized with the value of *gyrA*, which was constant in different growth conditions and phases (data not shown).

RESULTS

Optimization of experimental conditions for the transcriptome analysis

Four different types of peptides were chosen to study the response to antimicrobial peptides. Human cathelicidin LL-37 and porcine protegrin PG-1 are cathelin-associated α-helical and β-sheet structures, respectively (Turner *et al.*, 1998), and human defensin HNP-1 belongs to α-defensins with a triple-stranded β-sheet structure (Lehrer & Ganz, 2002). PLL was chosen as a synthetic analogue of cationic peptides (Vaara & Vaara, 1983). These antimicrobial peptides were first used at various concentrations in a Bioscreen assay to determine the range of concentrations needed to inhibit the growth of *B. subtilis*. Then, the effects of peptide concentrations that inhibited growth but did not kill bacteria were determined in shake-flask cultures (Fig. 1). Bacteria were grown in BFA minimal medium containing an additional 100 mM NaCl to enhance the microbicidal effect of the peptides (Turner *et al.*, 1998). No effect on growth was detected with HNP-1 for any of the tested concentrations (up to 9 μg ml^{-1}) and it was omitted from further analyses. For RNA isolations, bacteria were cultured in shake flasks and the antimicrobial peptides were added at 60 Klett units at concentrations of 1·5 μM (LL-37), 50 nM (PG-1) or 1 mM (PLL).

Cationic antimicrobial peptides induce a subset of SigW- and SigM-regulated genes

We studied the effects of the antimicrobial peptides on gene expression in *B. subtilis* using DNA macroarrays containing all the ORFs of the *B. subtilis* genome. Bacteria were exposed to peptide stress for 20 min, after which total RNA was isolated and the DNA macroarray analysis was carried out. In parallel, control cultures without a peptide addition were similarly treated. Two independent array experiments from separate cultures with each peptide treatment were performed. The array data were analysed with the ArrayVision and Microsoft Excel programs. Genes with a twofold induction ratio or higher and signal-to-noise ratios >3 in both independent experiments were considered to be induced.

Altogether, the LL-37 treatment induced 96 genes (Table 2, only the first gene of an operon is listed), including several genes that are regulated by the SigW and SigM ECF sigma factors (Huang *et al.*, 1999; Cao *et al.*, 2002a; Thackray & Moir, 2003; Asai *et al.*, 2003). Of the 30 verified promoters of

Table 1. Primers used in real-time PCR analyses

Target gene	Forward primers	Reverse primers
bceA	5′-cgagcattattttcgctgatga-3′	5′-taatagatcggaggccgatttc-3′
bcrC (*ywoA*)	5′-tcgttgcgatacagtgcat-3′	5′-tcgtatggtcacttggaaatgaa-3′
gyr	5′-gattattaacctgctggaggtagaaaa-3′	5′-aggtaaagctccgcattgaattc-3′
racX	5′-tccgcatcccgcaaaa-3′	5′-tggtagatggcggattgga-3′
radC	5′-acaagacggctgtttgaatgc-3′	5′-tccccgatcaccaaatgg-3′
wprA	5′-catctctgctccagggtctgata-3′	5′-ttccgctcatgtacgtgacatt-3′
yjbC	5′-tgtttgcggaattcgattctt-3′	5′-cctgccctcttgcatcctt-3′
ypuA	5′-ccgggactgccgcttta-3′	5′-tcctccgagatcgcttcgt-3′
yuaG	5′-ccgatgcagaccgttattctg-3′	5′-cgcgagctgtttggcttt-3′
yvcP	5′-tgcgccgcacatatggt-3′	5′ -ccgccaagctccactattct-3′
yvcR	5′-gcttgtgccagagcgattatt-3′	5′-cgaggtttccgttcggttca-3′
liaH (*yvqH*)	5′-tccgcgcgaattattcaaa-3′	5′-cgtctgcgaattctttacgtgta-3′
liaI (*yvqI*)	5′-catctgctcacttccgtttgtc-3′	5′-ttccagccgtaatacaccatagc-3′
yxdJ	5′-tgtatatcacgcggctcagaa-3′	5′-cgcaccgcttcaatagaaaca-3′
yxdL	5′-ggtcattcataagccgtcactca-3′	5′-ttgtcgctttggaatcaaggt-3′
yxdM	5′-tcattgccgtcatgcataca-3′	5′-cccgctacatcggaatatcct-3′

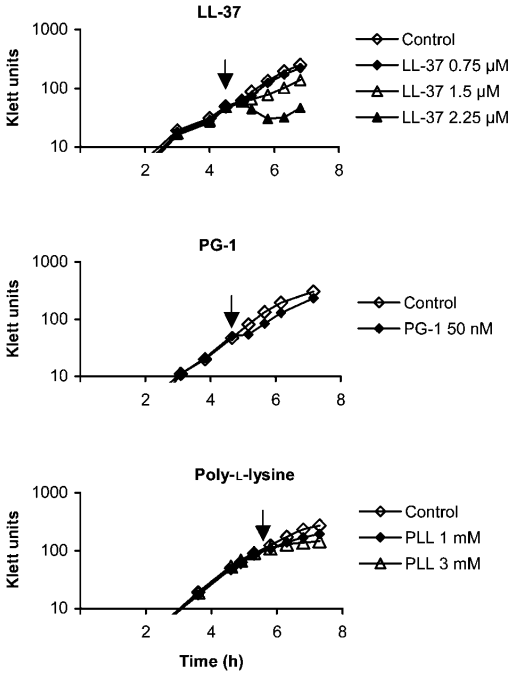

Fig. 1. The cationic peptides LL-37, PG-1 and PLL inhibit the growth of *B. subtilis*. Bacteria were grown in BFA minimal medium in shake flasks, and culture densities were measured with a Klett colorimeter. The cationic peptides were added at the cell density of 60 Klett units (arrow).

the SigW regulon (Cao *et al.*, 2002a), only 10 were expressed at elevated levels (greater than twofold) in LL-37-treated cells, with the fold-induction ratios ranging from 2·4 to 14·7, suggesting that LL-37 is a weak SigW inducer. The promoters that are directly regulated by SigM are less well known, but it was observed that 15 candidate promoters of the SigM regulon (Asai *et al.*, 2003), including *sigM* itself, were upregulated by LL-37 (Table 2). Many of the induced genes are involved in extracytoplasmic functions such as synthesis of the cell wall. These genes included *pbpE*, encoding a penicillin-binding protein (4·5-fold induction), *wapA*, encoding a cell wall-associated protein (2·5-fold induction), *murG*, involved in cell wall formation (6·1-fold-induction) and *maf*, required for septum formation (2·5-fold induction). The upregulated genes also included *bcrC* (*ywoA*), which is dependent on several sigma factors and is required for bacitracin resistance (Cao & Helmann, 2002) (4·1-fold induction), and the gene encoding the penicillin-binding protein *ponA* (2·3-fold induction). The most strongly induced gene was *liaI* (*yvqI*) (Mascher *et al.*, 2004), which was induced 58-fold (Table 2); other genes of the *liaIHGFSR* (*yvqIHGFEC*) gene cluster were also upregulated, but to a lesser extent (not shown). *liaI* (*yvqI*) is not known to be dependent on any ECF sigma factor. Furthermore, *yrhH* (14·7-fold), encoding a putative methyltransferase, *yxdL* (22·7-fold) and *yhcG* (14·7-fold), encoding putative ATP-binding components of ABC transporters, and *yoeB* (9·1-fold), encoding a putative exported protein of unknown function, were strongly upregulated.

The second natural peptide, PG-1, induced 58 genes. In a similar manner to LL-37, PG-1 also activated a subset of the genes of the ECF regulons (Table 2). Twelve genes of the SigW regulon, for which there are 30 verified promoters, and five genes predicted to belong to the SigM regulon (Asai *et al.*, 2003) were upregulated. The gene induction pattern resembled that of LL-37, but the induction ratios were lower.

Genes that were induced by both peptides were *araE*, *bcrC* (*ywoA*), *dltB*, *liaI*, *murG*, *pbpE*, *pspA*, *spoOM*, *wprA*, *yceC*, *yeaA*, *yjbC*, *yoeB*, *yqeZ* and *yuaG* (Table 2). Interestingly, the genes of the *yxdLM* operon, *yrhH* and *yhcG*, which were highly induced by LL-37, were not induced by PG-1 (see also below).

PLL was also an ECF inducer and the gene induction pattern resembled that of the other antimicrobial peptides, but characteristic differences were also observed (Table 2). Among 86 upregulated genes, there were 23 and 8 genes that belonged to the SigW and SigM regulons, respectively. Thus, compared to the response to LL-37 (above), it seems that PLL is clearly a stronger activator of the SigW regulon. There were also several genes that were induced at high levels by PLL, but not at all by LL-37 or PG-1, including *csfB* (6·6-fold), *yaaN* (5·1-fold), *yfhL* (5·6-fold), *yocA* (4·5-fold) and *yrzI* (12·4-fold), encoding proteins of unknown function. In addition, genes involved in purine, pyrimidine and ribosomal protein synthesis were strongly induced, a phenomenon not seen with the natural peptides. Furthermore, some genes that were induced at high levels by either or both of the natural peptides were not induced by PLL, notably *liaI* (*yvqI*) and other genes of the *lia* (*yvq*) cluster, *yxdL*, which was strongly induced by LL-37, and *yhcG* and *yoeB*.

Decreased expression of several genes was also observed. However, the experimental setup, short time of exposure to the peptides, and very different degradation rates of mRNAs hampered the interpretation of these results and they were not analysed in this study.

Cross-talk in signal transduction pathways mediating stress responses induced by a cationic peptide

We also carried out the DNA array analysis with *sigM* and *sigW* knockout mutants using LL-37 for the induction. The gene induction patterns of the sigma mutants and wild-type strain were compared in scatter plots (Fig. 2). The LL-37 treatment elicited a significantly lower number of induced genes in both sigma mutants than in the wild-type. The genes induced in the *sigM* and *sigW* mutants are listed in

Table 2. Genes induced by LL-37, PG-1 and PLL

Gene*	ECF sigma factor regulator†	Fold induction‡		
		LL-37	PG-1	PLL
abh	M,W,X		2·0 (0·5)	
araE		3·4 (±0·3)	6·3 (2·1)	4·4 (±0·9)
bcrC	M,V,W,X	4·1 (±0·6)	2·5 (0·5)	3·0 (±0·1)
citB		2·4 (±0·3)		
clpE			2·5 (0·6)	
codY		2·4 (±0·1)		
csbD			2·1 (0·8)	
csfB				6·6 (±3·1)
ddlA		2·7 (±0·4)		
dhbE		2·9 (±0·3)		
divIC	M,V,W,X	2·5 (±0·2)		
dltA§	V,W,X	¶	2·0 (0·5)	¶
fur				2·6 (±0·3)
glpD		2·4 (±0·4)		
greA	M	2·5 (±0·1)		
guaC				5·8 (±2·4)
iolS		2·3 (±0·2)		
liaI§		58·1 (±24·1)	15·3 (1·1)	
ligA	M,W	2·2 (±0·1)		
maf§	M,V,W	2·5 (±0·1)		
mrgA			2·7 (1·3)	
murE				2·4 (±0·2)
murG	M,W,Y	6·1 (±0·3)	2·3 (0·5)	
nfrA		3·3 (±0·4)		
parC		2·3 (±0·1)		
pbpE	W	4·5§ (±0·8)	3·5§ (0·5)	2·6 (±0·1)
pbpX	W,X		2·5 (0·3)	
phoA	M	4·5 (±1·8)		
ponA	M	2·3 (±0·3)		3·1 (±0·1)
pspA	V,W	2·7 (±0)	2·3 (0·8)	3·5 (±0·3)
pucJ				2·6 (±0)
purE				4·0 (±0·3)
pyrB§	W			18·7 (±3·8)
rplT				2·9 (±0·1)
rpmC§	V,W			2·2 (±0·2)
ruvB		5·1 (±3·0)		
sigI				6·6 (±1·3)
sigM§	M,W	3·4 (±0·7)		4·3 (±1·8)
sigW	W		2·3 (0·2)	5·1§ (±0·6)
sigX§	X		2·3 (1·1)	
speD				2·2 (±0·1)
spoOM	W	4·8 (±1·4)	2·7 (0·5)	3·6 (±0·2)
tagA				3·8 (±1·3)
tagG	W	4·6 (±0·9)		
trxB		2·5 (±0·5)		
wapA§	W	2·5 (±0·5)	¶	
wprA		2·6 (±0)	3·5 (0·7)	
yaaK		2·6 (±0·3)		
yaaN§	W			5·1 (±1·1)
ybcH				2·5 (±0·1)
ybyB			2·2 (0·5)	
yceC§	M,W,X	3·5 (±0·1)	2·1 (0·2)	3·7 (±0·9)

Table 2. cont.

Gene*	ECF sigma factor regulator†	Fold induction‡		
		LL-37	PG-1	PLL
ycnD		2·1 (±0·1)		
ydbO	M,V,X	6·0 (±1·2)		
ydbS§	W		2·3 (0·8)	3·9 (±0·1)
ydiP		3·2 (±0)		
yeaA	W	2·4 (±0·2)	2·1 (0·6)	5·4§ (±0·2)
yetG		3·8 (±0·5)		
yfhK			5·6 (2·8)	
yfhL§	W			5·6 (±0·9)
yfhM			2·3 (1·5)	
yflT			2·4 (1·6)	
yhaS		2·7 (±0·4)		
yhbB		3·5 (±0·6)		
yhcG		14·7 (±3·4)		
yhcU		2·3 (±0)		
yheN			2·1 (0·5)	
yjbC§	M,W,X	4·4 (±1·2)	2·9 (0·2)	3·8 (±0·5)
yjoB	W			4·2 (±0·5)
yknW	W			2·9 (±0·5)
yknZ	W		2·1 (0·6)	
ykoJ			3·3 (0·2)	
ykoK				2·9 (±0·6)
ykpC§				6·0 (±0·7)
yktC		2·4 (±0·2)		
ykvE				2·5 (±0)
ykvS		2·6 (±0)		
yndN	W			6·0 (±0·3)
yoaF	W			6·7 (±3·6)
yobJ	W			2·9 (±0·3)
yocA				4·5 (±1·0)
yocH	W			2·7 (±0·1)
yodT				2·2 (±0)
yoeB		9·1 (±1·6)	3·9 (1·1)	
yojG		2·5 (±0·3)		
yozO	W			2·9 (±0·7)
ypbH		2·8 (±0·3)		
ypuA		5·7 (±0·1)		2·9 (±0·8)
ypuD		3·5 (±1·1)		
ypwA		2·5 (±0)		
yqeZ§	V,W	2·5 (±0·4)	3·5 (0·7)	5·0 (±2·1)
yrbC		3·4 (±0·7)		
yrhH	M,W,X	14·7 (±2·1)		4·6 (±0·4)
yrhJ	M	3·6 (±0·1)		
yrkN				4·2 (±1·7)
yrrM§	V,W			2·9 (±0·1)
yrzG		2·6 (±0·4)		
yrzI				12·4 (±7·1)
ysdA				3·1 (±0·6)
ysdC		2·5 (±0·2)		
ytcJ		2·5 (±0·4)		
ythP§	W			3·8 (±0·8)
ytiP				2·3 (±0·1)
ytrF		2·6 (±0·4)		

Table 2. cont.

Gene*	ECF sigma factor regulator†	Fold induction‡		
		LL-37	PG-1	PLL
ytzB		3·5 (±1·1)		
yuaD		2·3 (±0·2)		
yuaF§	W	¶	5·9 (0·5)	3·4 (±0·1)
yugP		2·4 (±0·1)		
yuxN		5·6 (±0·5)		
yvcR§		3·5 (±0·6)		
yvgN		2·8 (±0·2)		
yvlA§	W			3·4 (±0·8)
yvyE				4·3 (±2·1)
ywaC	M,W			4·4 (±0·8)
ywiE			2·9 (1·1)	
ywjC			2·1 (0·7)	
ywnF		2·4 (±0·3)		
ywrE	W		2·4 (0·1)	7·1 (±2·5)
ywrO		2·6 (±0·4)		
ywsB			2·4 (0·7)	
ywzA			2·1 (0·6)	
ywzC				2·1 (±0)
yxdL§		22·7 (±9·1)		
yxiE	M	2·9 (±0·3)		
yxjI	W	3·4 (±0·2)		6·5 (±0·2)
yxkD				3·2 (±0·2)
yxkH	Y		2·6 (1·3)	
yyaK			2·3 (0·1)	
yybD			2·0 (1·2)	

*Only the first gene of an operon is listed.

†Evidence suggesting regulation by ECF sigma factors has previously been reported (Huang & Helmann, 1998; Huang *et al.*, 1999; Wiegert *et al.*, 2001; Cao *et al.*, 2002a; Asai *et al.*, 2003; Cao & Helmann, 2004). Underlining indicates a verified promoter (Huang & Helmann, 1998; Cao *et al.*, 2002a; Cao & Helmann, 2004). The ECF sigma factor dependencies of other genes may include indirect effects.

‡The ratio of the expression level of the gene in treated cells to that in untreated cells, as determined by DNA macroarray. Mean of two or three experiments. The standard deviation or range is shown in parentheses.

§Whole operon was induced.

¶Downstream genes were induced.

Table 3. This reduced stress response was not due to decreased stress in the sigma mutants, since the effective concentration of LL-37 causing the growth inhibition was the same in all three strains (see below). In the *sigM* mutant, as expected, the genes of the SigM regulon were not induced. Surprisingly, the array data also showed either that the SigW-regulated genes were not induced or that their induction levels were clearly lower than in the wild-type strain. Analogous results were obtained with the *sigW* mutant. Interestingly, numerous genes which are not known to belong to these two sigma factor regulons were also induced in a SigM- or SigW-dependent manner. These results suggest significant cross-talk between the SigM and SigW regulons, and some other regulon(s) responding to LL-37. Furthermore, a significant observation was the clearly higher level of expression of several genes in the *sigW* mutant compared to that of the wild-type (Fig. 2b). This set included (Table 3) genes encoding endo-1,4-β-glucanase (*bglC*; 4·6-fold), a protein synthesizing α-1,4-glucan using ADP-glucose (*glgA*; 5·1-fold) and 6-phospho-α-glucosidase (*glvA*/*malA*; 3·9-fold).

In order to study the overlap between the stress regulons further, we determined the expression levels of some of the induced genes by real-time RT-PCR. The *liaI* and *yxdL* genes were chosen due to their strong induction with LL-37. The *yuaG* gene (in the *yuaFGI* operon) was induced most strongly by PG-1. It encodes a putative flotillin known to belong to the SigW regulon (Huang *et al.*, 1999; Cao *et al.*, 2002a). Other chosen genes included *racX* (in an operon

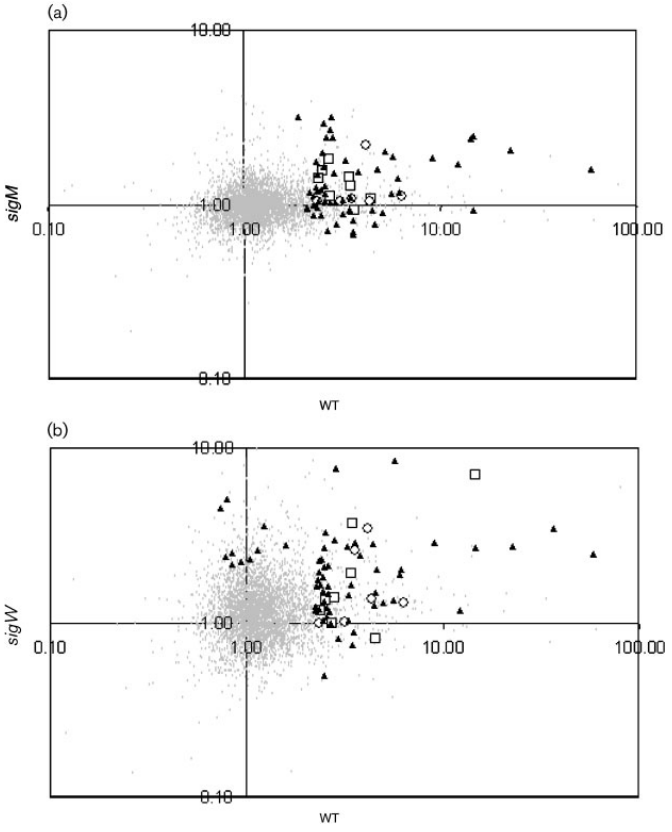

Fig. 2. Comparison of the induction ratios of the wild-type strain and the *sigM* (a) and *sigW* (b) mutants treated with LL-37. The genes induced in the wild-type and/or a sigma mutant by LL-37 are highlighted (closed triangle, open circle or square). The genes belonging to the SigM or SigW regulons are marked with open circles and squares, respectively. The induction ratios of other genes are marked with grey circles and include some high induction ratios due to the use of non-filtered data in this graphical comparison; the filtering (see Methods) of data eliminated these high induction ratios. WT, wild-type.

with *pbpE*), a SigW-dependent gene (Cao *et al.*, 2002a), and *yjbC* and *bcrC* (*ywoA*), which belong to the SigM regulon (Cao & Helmann, 2002; Ohki *et al.*, 2003a). The *yjbC* gene is also regulated by SigW and SigX (Cao *et al.*, 2002a; Ohki *et al.*, 2003a; Thackray & Moir, 2003). Furthermore, the expression of *radC* (in an operon with *maf*), which may be regulated by SigM (Asai *et al.*, 2003), and of *wprA* and *ypuA*, which may be expressed independently of the ECF sigma factors (Table 2), was determined. The RT-PCR analysis was carried out with the wild-type strain and the *sigM* and *sigW* mutants. A *sigM sigW* double mutant was also used to analyse the expression of some genes. Bacteria were treated in a similar manner to that employed in the array experiments with LL-37 or PG-1. Since the array analysis revealed that the induction of *radC* and *ypuA* with PG-1 was low, their induction with this peptide was not determined. Samples were collected for analysis at two different time points, 10 and 20 min after the addition of the antimicrobial peptides. All mRNA measurements were performed two to four times.

In the wild-type strain treated with LL-37, *liaI* and *yxdL* were the most highly induced genes (Table 4), as was also the case in the array analysis. Higher induction ratios were seen in the 10 min samples than in the 20 min samples, indicating that the induction was fast and transient. As shown in Fig. 3, expression was dependent on the dosage of LL-37, as demonstrated with the *liaI* gene (Fig. 3a), and decreased (also that of *liaH*) from the maximal level (10 min time point) back to the uninduced level in about 2 h (Fig. 3b). Consistent with the induction of the *liaIHGFSR* gene cluster, the LiaH protein appeared in the proteome of cytoplasmic proteins, as demonstrated by two-dimensional gel electrophoresis and spot identification by matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) (data not shown). The other genes were induced to a lesser extent, and in only half of them was the induction transient (Table 4). The normalized mRNA levels (not shown) indicated that *liaI*, *yxdL* and *yuaG* were expressed at a very low level in non-treated cells (basal expression level). The basal expression level of *wprA*, *yjbC*, *ypuA* and *radC* was fairly high, and *bcrC* (*ywoA*) and *racX* were expressed at intermediate levels. The genes that were expressed at a low level in non-treated cells exhibited the strongest induction in peptide-treated cells. Normalized mRNA levels of the genes varied approximately

Table 3. Induction of gene expression in cells of the *sigW* and *sigM* mutants treated with LL-37

Gene	Fold induction*		
	sigW	*sigM*	WT†
bcrC	3·5 (±0·2)		4·1 (±0·6)
bglC	4·6 (±1·2)		
cydC		2·4 (±0·2)	
dltB	2·3 (±0·2)	3·2 (±0·4)	2·8 (±0·4)
dltC		2·9 (±0·4)	2·5 (±0·4)
ftsY	2·3 (±0)		
glgA	5·1 (±1·5)		
glvA	3·9 (±1·2)		
liaI	2·4 (±0·4)		58·1 (±24)
minC	3·5 (±0·1)		2·3 (±0·2)
qoxC	2·5 (±0·5)		
sigX		2·4 (±0·2)	2·6 (±0·6)
wapA		2·9 (±0·1)	2·6 (±0)
yceC	3·7 (±0·5)		3·5 (±0·1)
yfiJ	3·6 (±1·4)		
yhcH	2·4 (±0·1)		4·6 (±1·2)
yisV	2·4 (±0)		
ykfA	2·1 (±0)		
ypbH	5·8 (±3·8)		2·8 (±0·3)
ypuA	8·4 (±1·9)		5·7 (±0·1)
yrhH	7·0 (±3·3)		14·7 (±2·1)
yrhJ	2·9 (±0·7)		3·6 (±0·1)
yrvP	2·6 (±0·1)		
yrzG		2·8 (±0·4)	2·6 (±0·4)
ytrF	2·8 (±0·5)		4·4 (±0·7)
yvgN	7·6 (±4·7)		2·8 (±0·2)
ywaC	4·0 (±0·1)		7·4 (±1·8)
ywdA		2·0 (±0)	
yxdL	2·7 (±0·5)		22·7 (±9·1)
yxeA	3·5 (±0·4)		36·4 (±2·1)
yxiG	3·4 (±0·1)	2·7 (±0)	2·4 (±0·2)
yxjF	2·2 (±0·2)		
yxkH	3·0 (±0·3)	2·4 (±0·2)	
yxxG	4·5 (±0·9)	3·4 (±0·8)	3·6 (±0·6)
yxzC	3·5 (±0·2)	3·2 (±0·2)	3·6 (±0·1)
yxzG	2·5 (±0·2)		2·9 (±1·0)
yyaK		3·2 (±1·1)	

*The ratio of the expression level of the gene in treated cells to that in untreated cells, as determined by DNA macroarray. Mean of two experiments. The range is shown in parentheses.

†The induction ratios of all genes (or the first genes of operons) induced in a wild-type (WT) strain (LL-37 treatment) are shown in Table 2.

twofold from one experiment to another and between non-treated wild-type and ECF sigma mutant cells (not shown). Considerable experimental variation was especially observed in the induction ratios of the *liaI* and *yxdL* genes (Table 4). The RT-PCR displayed clearly higher induction ratios than the DNA array, but there was a good overall consistency of the induction pattern in these two types of assay.

The *liaI* gene, which was induced 1530-fold in the wild-type (10 min time point) with LL-37, was also upregulated in the *sigM* and *sigW* mutants, but in both of them the induction was clearly lower, 198- and 287-fold, respectively (Table 4). In DNA arrays, the difference in the induction ratios was more dramatic, 58-fold in the wild-type and twofold in the mutants. The other highly induced gene, *yxdL*, was also upregulated significantly less in the *sigM* (248-fold) and *sigW* (347-fold) mutants compared to the wild-type strain (704-fold), consistent with the array results above. Only a minor additive effect on *liaI* and *yxdL* expression was observed in the double mutant (Table 4). The *yuaG* (*yuaFGI*) gene was induced 18-fold (10 min time point), whereas no induction was seen in the *sigW* mutant, consistent with its previously verified SigW dependency (Cao *et al.*, 2002a) (see also below). The induction of *yuaG* was also reduced in the *sigM* mutant (sevenfold at the 10 min time point), suggesting moderate SigM dependency. The *yjbC* and *bcrC* (*ywoA*) genes were induced in the wild-type strain 6–11-fold, whereas in the *sigM sigW* double mutant almost no induction was seen (Table 4), indicating that these genes are induced in an ECF-dependent manner. However, the inactivation of single ECF sigma factors reduced the induction ratios only moderately or not at all. LL-37 induced *yjbC* expression three- to fivefold in the *sigM* mutant and six- to ninefold in the *sigW* mutant. The induction ratios of *bcrC* were 3–6 (*sigM*) and 6–9 (*sigW*). *racX* induction was affected by both sigma mutations (see also the effects of PG-1 and Triton X-100 on *racX* expression below). These results suggest that both SigM and SigW regulate either directly or indirectly the *yjbC*, *bcrC* (*ywoA*) and *racX* genes. LL-37 upregulated the expression of *radC*, *wprA* and *ypuA* four- to 12-fold in the wild-type. Reduced induction ratios of *radC* and *ypuA* in the *sigM* mutant suggest that their induction by LL-37 is at least partially mediated by SigM (Table 4). The *wprA* gene exhibited induction patterns that rather suggest the independence of the sigma factors (see also the effects of PG-1 and Triton X-100 on *wprA* expression below).

The induction ratios of all genes studied were significantly lower in PG-1-treated cells than in LL-37-treated cells (Table 5), suggesting that the stress caused by the PG-1 treatment was less severe; for example, the induction ratio of *liaI* with LL-37 was several-fold higher than with PG-1. Despite the lower induction level in PG-1-treated cells, the *sigM* and *sigW* mutations reduced the induction of *liaI* as in LL-37-treated cells (Table 5). The concentration of PG-1 used induced the *yjbC* and *bcrC* (*ywoA*) genes very weakly in all three strains. PG-1 treatment induced *yuaG* expression in a similar manner to LL-37 treatment, and the strong SigW dependency and moderate SigM dependency of *yuaG* were also observed with PG-1 (Table 5). In the wild-type, *racX* was induced four- to ninefold, whereas no induction was observed in the *sigW* mutant, suggesting SigW-dependent regulation of *racX*. In the *sigM* mutant, *racX* was induced two- to sixfold by PG-1. The inactivation of the sigma factors did not impair the induction of *wprA*,

Table 4. The dependency of a set of LL-37-induced genes on SigM and SigW

Gene	Fold induction*											Function of encoded protein
	WT			Mutation†								
				sigW			*sigM*			*sigW*	*sigM*	
	10 min	20 min	Array	10 min	20 min	Array	10 min	20 min	Array	10 min	20 min	
bcrC	9·9 (0·9)	6·0 (2·5)	4·1	9·2 (5·2)	6·1 (2·7)	3·5	3·2 (0·8)	6·1 (0·9)	5·9	1·8 (±0·2)	2·1 (±0·2)	Bacteriocin transport permease
racX	6·0 (2·5)	6·9 (3·5)	5·0	1·5 (0·5)	2·4 (0·6)	1	3·2 (1·6)	2·8 (0·7)	1·6	1·4 (±0·1)	2·3 (±0·1)	Amino acid racemase
radC	6·3 (±1·1)	3·9 (±0·8)	2·4	3·4 (±3·4)	2·8 (±0·2)	0·9	1·3 (±0·1)	1·5‡	1·8			DNA repair protein
wprA	6·4 (1·8)	5·6 (2·1)	2·6	3·2 (1·2)	2·9 (0·2)	1·7	6·1 (2·5)	6·4 (1·1)	3·6	2·4 (±0·1)	3·4 (±0·2)	Cell wall-associated protein precursor
yjbC	11·5 (2·9)	9·1 (3·0)	4·4	9·0 (7·2)	6·4 (4·7)	1·4	3·0 (0·8)	4·7 (1·7)	1	1·1 (±0)	1·4 (±0·29)	Unknown
ypuA	10·4 (±0·2)	12 (±4·4)	5·7	11·0 (±2·6)	11·6 (±0·8)	8·4	2·6 (±0·5)	4·2‡	2·6			Unknown
yuaG	17·7 (±4·5)	7·4 (±0)	2·7	1·3 (±0·4)	1·1 (±0·1)	1	6·9 (±0·1)	3·5‡	2·1			Similar to flotillin 1
liaI (*yvqI*)	1530 (860)	610 (560)	58·1	287 (220)	51 (45)	2·4	198 (200)	54 (69)	1·7	106 (±41)	37 (±16)	Unknown
yxdL	704 (113)	338 (63)	22·7	347 (219)	126 (90)	2·7	248 (48)	49 (15)	2·3	123 (±17)	29 (±5·2)	Similar to ABC transporter

*The ratio of the expression level of the gene in LL-37-treated cells to that in untreated cells, as determined by RT-PCR. The ratios from the DNA array analyses, if determined (Table 2), are also shown. The standard deviation or range is shown in parentheses. WT, wild-type.
†The *sigW*::*neo* (constructed in the laboratory of J. D. Helmann) and *sigM*::pMUTIN4 (Horsburgh & Moir, 1999) knockout mutations. The *sigW sigM* double mutant contains both of them.
‡Only one measurement.

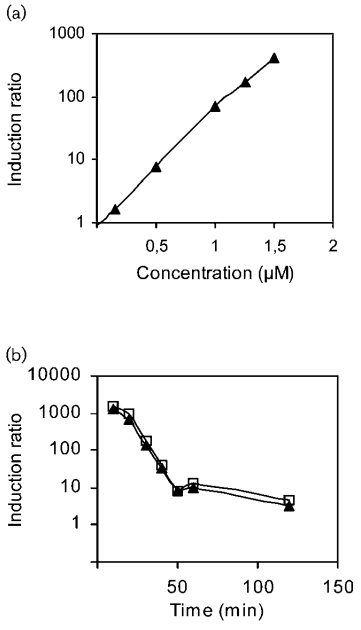

Fig. 3. Dependence of *liaI* expression on the concentration of LL-37 and kinetics of LL-37-induced expression of *liaI* and *liaH*. Bacteria were grown in BFA minimal medium. (a) The induction of *liaI* in cells stressed with various concentrations of LL-37. (b) LL-37 (1·5 μM) was added at the cell density of 60 Klett units and the expression of *liaI* (closed triangle) and *liaH* (open square) was determined by RT-PCR at various time points of the LL-37 treatment. The induction ratio is fold induction in LL-37-treated cells compared to that in non-treated cells.

consistent with an induction mechanism that is independent of SigW and SigM.

Triton X-100 induces sigma regulons, but in a pattern different from that of antimicrobial peptides

In order to find out whether the above genes are also induced by a membrane-disrupting agent with no presumed specificity, we treated cells with 0·005 % Triton X-100 and determined expression levels of the genes in the wild-type strain and the ECF sigma factor mutants by real-time RT-PCR. The effects of cationic peptides on membranes may be

Table 5. Induction of a set of genes by PG-1 and their dependence on SigM and SigW

Gene	Fold induction*							Function of encoded protein
	WT			Mutation†				
				sigW		*sigM*		
	10 min	20 min	Array	10 min	20 min	10 min	20 min	
bcrC	2·0 (±0·1)	3·3 (±0·2)	2·5	1·7 (±0·1)	2·2 (±1·0)	1·2 (±0·1)	5·3 (±0·2)	Bacteriocin transport permease
racX	4·3 (±1·1)	8·7 (±0·1)	3·8	1·1 (±0)	1·3 (±0·1)	2·5 (±0·1)	5·9 (±0·7)	Amino acid racemase
wprA	1·4 (±0·1)	3·9 (±0·7)	3·5	1·9 (±0·1)	3·8 (±0·5)	2·3 (±0·2)	4·1 (±0·3)	Cell wall-associated protein precursor
yjbC	1·3 (±0·3)	2·6 (±0·4)	2·9	1·6 (±0·1)	1·8 (±0·2)	2·6 (±0·2)	2·3 (±0·2)	Unknown
yuaG	17·1 (±0·5)	9·7 (±0·4)	6·8	0·5 (±0·2)	0·5 (±0·1)	6·4 (±0·1)	4·3 (±0·9)	Similar to flotillin 1
liaI (*yvqI*)	328 (±34)	258 (±36)	15·3	118 (±2)	59 (±11)	13·3 (±3·3)	13·2 (±2·4)	Unknown
yxdL	1·5 (±0·1)	1·4 (±0)	1·4	0·9 (±0·1)	0·9 (±0·1)	1·1 (±0·1)	1·1 (±0·2)	Similar to ABC transporter

*The ratio of the expression level of the gene in PG-1-treated cells to that in untreated cells, as determined by RT-PCR. The ratios from the DNA array analyses, if determined (Table 2), are also shown. The range is shown in parentheses. WT, wild-type.
†The *sigW*::*neo* (constructed in the laboratory of J. D. Helmann) and *sigM*::pMUTIN4 (Horsburgh & Moir, 1999) knockout mutations.

somewhat different from those of detergents (Henzler Wildman *et al.*, 2003), although the opposite view has also been put forward (Oren *et al.*, 1999).

A similar induction of *liaI* expression (790-fold at the 10 min time point) and slightly reduced induction ratios in the *sigM* and *sigW* mutants, as with LL-37, were observed (Table 6). The strong dependency of *yuaG* on SigW was also demonstrated with the detergent, but the moderate SigM dependency was not observed. The induction of three other genes (*radC*, *ypuA* and *bcrC*) was also partially dependent on both sigma factors. *radC* was expressed in the wild-type strain at two- to fivefold, *ypuA* at two- to threefold and *bcrC* (*ywoA*) at threefold higher levels than in the sigma mutants (Table 6). In the wild-type strain and *sigM* mutant, a clearly stronger induction of *racX* was observed when cells were treated with Triton X-100 (19–61-fold) than when they were treated with the antimicrobial peptides (four- to ninefold). In a similar manner to that observed with PG-1, no induction was seen in the *sigW* mutant, consistent with the SigW dependency of *racX*. The sigma mutations did not impair the induction of *yjbC* in Triton X-100-treated cells, in contrast to LL-37-treated cells. Furthermore, the *wprA* gene was not induced by Triton-X-100.

Table 6. Induction ratios in cells treated with Triton X-100

Gene	Fold induction*						Function of encoded protein
	WT		Mutation†				
			sigW		*sigM*		
	10 min	20 min	10 min	20 min	10 min	20 min	
bcrC	6·3 (±1·4)	9·7 (±2·8)	2·4 (±0·2)	2·7 (±0·4)	1·9 (±0)	2·8 (±0·1)	Bacteriocin transport permease
racX	40·4 (±1·6)	61·4 (±9·4)	1·0 (±0·1)	0·8 (±0)	19 (±1·5)	26·3 (±3·5)	Amino acid racemase
radC	3·9 (±0·3)	5·0 (±0·5)	1·6 (±0)	1·6 (±0·1)	1·2 (±0·3)	0·8 (±0·1)	DNA repair protein
wprA	1·3 (±0·1)	2·0 (±0·2)	1·4 (±0·2)	1·4 (±0·1)	1·9 (±0·1)	2·6 (±0·1)	Cell wall-associated protein precursor
yjbC	4·6 (±0·9)	5·1 (±0·3)	5·8 (±0·1)	7·2 (±0·7)	11·9 (±0·2)	5·1 (±0·2)	Unknown
ypuA	6·9 (±0·6)	10·1 (±0·4)	2·1 (±0·1)	3·5 (±0·1)	3·7 (±0·3)	2·9 (±0·1)	Unknown
yuaG	42·1 (±3·6)	19·4 (±8·4)	1·4 (±0·5)	1·2 (±0·2)	37·5 (±2·6)	12·8 (±0·1)	Similar to flotillin 1
liaI (*yvqI*)	790 (±276)	441 (±58)	347 (±48)	192 (±44)	375 (±77)	227 (±16)	Unknown
yxdL	1·0 (±0·1)	1·6 (±0·2)	1·4 (±0·2)	0·6 (±0)	1·5 (±0·1)	1·3 (±0·1)	Similar to ABC transporter

*The ratio of the expression level of the gene in cells treated with Triton X-100 to that in untreated cells, as determined by RT-PCR. The range is shown in parentheses. WT, wild-type.
†The *sigW*::*neo* (constructed in the laboratory of J. D. Helmann) and *sigM*::pMUTIN4 (Horsburgh & Moir, 1999) knockout mutations.

Inactivation of SigM and SigW does not increase the sensitivity of *B. subtilis* to antimicrobial peptides

The sensitivity of *sigW* and *sigM* mutants and the *sigM sigW* double mutant to LL-37, PG-1 and PLL was tested. Growth experiments were performed in shake-flask cultures in the same conditions as in the array experiments. The growth was also studied with several peptide concentrations with Bioscreen. No significant difference in the sensitivity of the *sigW*, *sigM* or *sigM sigW* mutants to the antimicrobial peptides was detected and the growth arrest caused by the antimicrobial peptides was similar to that of the wild-type. However, it was obvious that the *sigW* and *sigM sigW* mutants grew slower than the wild-type (in the absence of peptide) in the BFA minimal medium containing 100 mM NaCl, indicating the importance of the SigW regulon in these conditions (data not shown). The growth of the *sigM* mutant resembled that of the wild-type.

yxdL gene expression is specifically induced by LL-37

A highly interesting observation was that the *yxdL* gene, which was strongly induced by LL-37 (704-fold), was not induced by PG-1 (Table 5). The lack of induction was seen in both the DNA array and RT-PCR analyses, and it was true for both wild-type and *sigM* and *sigW* mutant cells. The *yxdL* gene was not induced by Triton X-100 either (Table 6). These results suggest that there is a strong specificity in the induction mechanism of *yxdL*.

LL-37 causes upregulation of three paralogous ABC-transporter genes via TCS-mediated signalling dedicated to the regulation of transporter expression

The *yxdL* gene encodes the putative ATP-binding component of an ABC transporter of unknown function. The downstream gene *yxdM*, which most probably forms an operon with *yxdL*, encodes the permease component of the ABC transporter. Immediately upstream from *yxdLM* there are the *yxdJ* and *yxdK* genes, which encode the components of a TCS of unknown function (see the organization of the gene cluster in Fig. 4). The *yxeA* gene, which encodes a conserved protein of unknown function, is located downstream from the *yxdM* gene and most probably belongs to the same operon as *yxdLM* (not shown in Fig. 4). The DNA array data also revealed that LL-37 strongly induced the *yxeA* gene (36-fold) in a similar manner to *yxdLM*, consistent with the operon organization. A sequence similarity search revealed that *yxdL* and *yxdM* are highly homologous with the corresponding genes of two other ABC transporters of *B. subtilis*, BceAB (formerly YtsCD) and YvcRS (Fig. 4; see also Joseph *et al.*, 2002; Mascher *et al.*, 2003; Ohki *et al.*, 2003b). Interestingly, genes encoding TCSs are also located in the immediate upstream regions of the *bceAB* and *yvcRS* genes in a pattern similar to that of the *yxdLM* region. It has been shown that the BceRS TCS is able to sense extracellular bacitracin and induce the expression of the BceAB ABC transporter, which confers resistance to bacitracin (Mascher *et al.*, 2003; Ohki *et al.*, 2003b). The YxdLM ABC transporter as well as the YxdJK TCS exhibit homology with the corresponding proteins of the Yvc and Bce systems. The homology is highest between the ATP-binding components of the ABC transporters (about 50 % identity), and, in the following order, is less between the response regulators of TCS, the sensor kinases of TCS and the permease components of the ABC transporters (Fig. 4). The Yxd proteins exhibit slightly higher similarity to the Yvc proteins than to the Bce proteins.

The DNA array analysis showed that the *yvcR* and *yvcS* genes were upregulated 3·5- and sixfold in LL-37-treated cells, respectively, suggesting that this antimicrobial peptide may affect not only *yxdL* expression but also its paralogues. The regulation of the three paralogous ABC transporter genes was studied by determining their expression in the wild-type strain and mutants of the upstream histidine kinase genes (*yxdK*, *yvcQ* and *bceS*) treated with LL-37 using real-time RT-PCR. In the wild-type strain, the *yxdM* gene was induced strongly (about 300-fold at the 10 min time point) in a similar manner to the *yxdL* gene (Table 7), further indicating that these genes form an operon (together with *yxeA*). Since no induction of the upstream *yxdJ* gene was observed, the TCS genes must belong to a different transcriptional unit than *yxdLM*, in a manner similar to that by which the *bceRS* genes are transcribed separately from *bceAB* (Joseph *et al.*, 2002; Ohki *et al.*, 2003b). In the *yxdK* mutant, hardly any induction of *yxdL* and *yxdM* was seen, indicating that the YxdJK TCS regulates the expression of the *yxdLM* operon (Table 7). The induction of *yxdL* was also slightly reduced (about 25 %) in both the *yvcQ* and the *bceS*

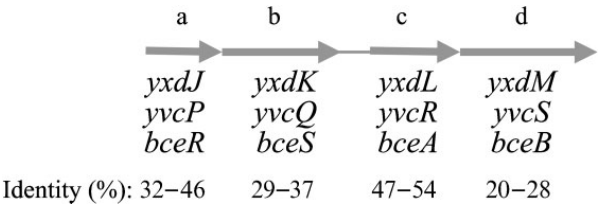

Fig. 4. Organization of three pairs of genes encoding homologous ABC transporters and in their immediate upstream region three similar pairs of genes encoding homologous TCSs. The identity values of the amino acid sequences of the deduced protein products are indicated. a, Response regulator of TCS; b, sensor histidine kinase of TCS; c, ATP-binding component of ABC transporter; d, permease component of ABC transporter.

Table 7. Expression regulation and cross-regulation of the *yxdLM* operon and its homologues *yvcRS* and *bceAB* via the YxdK, YvcQ and BceS TCSs

Gene	Fold induction*							
	WT		Mutation†					
			yxdK		*yvcQ*		*bceS*	
	10 min	20 min	10 min	20 min	10 min	20 min	10 min	20 min
bceA	6·1 (0·8)	3·8 (1·0)	8·1 (3·4)	3·4 (0·8)	7·0 (3·0)	3·6 (1·0)	3·3 (±0·4)	0·6 (±0·1)
yvcP	0·8 (0·2)	0·8 (0·2)						
yvcR	5·6 (0·5)	7·8 (4·6)	3·4 (1·2)	1·4 (0·4)	2·2 (0·6)	3·2 (0·5)	7·2 (±0·3)	3·2 (±0·1)
yxdJ	1·2 (0·4)	0·8 (0·3)						
yxdL	523 (228)	197 (12)	1·5 (±0·3)	1·8 (±0·1)	328 (142)	148 (53)	324 (±67)	148 (±34)
yxdM	320 (37)	239 (47)	1·4 (0·5)	2·6 (1·0)				

*The ratio of the expression level of the gene in LL-37-treated cells to that in untreated cells, as determined by RT-PCR. The standard deviation or range is shown in parentheses. WT, wild-type.

†Knockout mutations constructed with pMUTIN4 (Vagner *et al.*, 1998).

mutant, suggesting cross-regulation between the systems; in other words, the paralogous TCSs also regulate the promoters of the non-cognate paralogous ABC-transporter genes. The *yvcR* gene was induced by LL-37, but only about twofold, consistent with the DNA array result for *yvcRS* induction. The inactivation of the upstream sensor kinase gene *yvcQ* only partially abolished the induction of *yvcR* (2·2-fold induction at the 10 min time point). Similar partially abolished induction was also seen in the *yxdK* mutant (3·4-fold), consistent with cross-regulation (Table 7). In the *bceS* mutant, the induction of *yvcR* was comparable to that of the wild-type. LL-37 did not induce *yvcP* expression, indicating that *yvcPQ* and *yvcRS* are different transcriptional units. The DNA array results suggested that the *bceAB* operon is not induced by LL-37. The RT-PCR analysis, however, revealed that *bceA* is also induced about sixfold in LL-37-treated cells (Table 7). The *bceS* mutation partially abolished the induction (3·3-fold), but the *yvcQ* and *yxdK* mutations did not affect it.

In order to find out whether or not the YxdLM or YvcRS ABC transporters have a role in peptide resistance, the sensitivity of *yxdL* and *yxdL yvcR* mutants to LL-37 was tested. Neither the single mutant nor the double mutant showed increased sensitivity to LL-37.

DISCUSSION

In this study, we carried out genome-wide transcription analyses of the stress responses of *B. subtilis* to the cationic antimicrobial peptides LL-37 and PG-1, which are natural peptides, and PLL, a synthetic peptide. The responses to each peptide were highly complex, including activation of several signal transduction pathways.

The antimicrobial peptides induced expression of 96 (LL-37), 58 (PG-1) and 86 (PLL) genes in *B. subtilis*. In this complex response, some patterns were recognized. A prominent feature was a high proportion of induced genes belonging to the SigW and SigM ECF-type sigma factor regulons. However, only subsets of these sigma factor regulon genes were induced by the antimicrobial peptides. The non-amphipathic PLL was the most effective peptide in activating the SigW regulon, as evidenced by the induction of 23 out of the 30 verified promoters of the regulon [see Cao *et al.* (2002a) for the SigW regulon]. The amphipathic peptides LL-37 and PG-1 upregulated only about one-third of the 30 SigW-regulated genes. In a similar manner, only subsets of putative SigM-regulated promoters were induced in peptide-treated cells. These results suggest that SigW and SigM are involved in the stress responses to the antimicrobial peptides. However, the high numbers of induced genes that are expressed independently of SigW and SigM suggest that probably several other signal transduction pathways and regulators also mediate the stress responses.

It has been shown that the ECF sigma factor regulons are partly overlapping (Cao *et al.*, 2002b; Huang *et al.*, 1998). Nevertheless, the effect of *sigW* or *sigM* mutation on the number of induced genes in cells treated with LL-37 was striking. Not only the genes belonging to the mutated sigma regulon but also those under the control of other ECF sigma factors and those expressed independently of these sigma factors were poorly induced after peptide treatment. This phenomenon may be partly due to the increased basal expression level of several genes in the *sigW* and *sigM* mutants. Consequently, the additional stress of cationic peptides may not have caused further induction. Yet this alone does not explain why so few genes were induced in the sigma factor mutants, since the basal expression level was elevated in the sigma mutants only in the case of about 30 % of the genes induced in the wild-type. The RT-PCR analysis revealed decreased induction ratios for *bcrC*, *liaI*, *radC*, *racX*, *ypuA* and *yxdL* in both the sigma

mutants. However, it is apparent that not all the affected genes are directly regulated by the sigma factors. It has been shown that *liaI* is regulated by the LiaRS (YvqCE) TCS (Mascher *et al.*, 2003; H.-L. Hyyryläinen and others, unpublished results). The effects of ECF sigma factor mutations on *liaI* expression in cells treated with alkaline (Wiegert *et al.*, 2001) or cationic antimicrobial peptides (this study) are most probably indirect. It is possible that the inactivation of one ECF sigma factor results in disturbance of the sensory function of other membrane-associated stress sensors. The similarity of the lethal doses of the cationic peptides in the sigma factor mutants and the wild-type, however, suggests that the degree of stress in these strains was the same. These results suggest that the stress response to cationic antimicrobial peptides is very complex. The functional overlap of several sigma factors and other types of regulators may also explain why the inactivation of SigW, SigM or both of them did not make cells sensitive to antimicrobial peptides.

PLL is expected to interact with the negatively charged cell wall and head groups of the membrane phospholipids (carpet or detergent-like mechanism; Yeaman & Yount, 2003), but may not penetrate deeper into the membrane interior. LL-37 and PG-1 penetrate into the membrane and disturb its integrity by forming pores (Henzler Wildman *et al.*, 2003; Oren *et al.*, 1999; Yang *et al.*, 2000). We hypothesize that SigW-regulated promoters are activated by antimicrobial peptides by the interaction of the latter with the cell membrane surface and the cell wall, rather than by deeper effects inside the membrane. This conclusion is consistent with the strong induction of the SigW regulon by cell wall antibiotics (Cao *et al.*, 2002b).

The *araE*, *bcrC* (*ywoA*), *dltB*, *pbpE*, *pspA* (*ydjF*), *yceC*, *spo0M*, *yeaA*, *yjbC*, *yqeZ* and *yuaG* genes were induced by all three peptide treatments. Each of these genes belongs to at least one ECF sigma factor regulon (Table 2). Some of these genes are involved in interactions with antimicrobial compounds interfering with the cell wall or membrane: *pbpE* encodes a penicillin-binding protein, *yceC* is similar to the tellurium resistance proteins, and *bcrC* (*ywoA*) encodes a bacitracin permease (Cao & Helmann, 2002; Podlesek *et al.*, 1995). The *dlt* operon including *dltB* is involved in the D-alanine esterification of lipoteichoic and wall teichoic acids (Perego *et al.*, 1995), which increases bacterial resistance to cationic antimicrobial peptides (Peschel *et al.*, 1999; Cao & Helmann, 2004).

The DNA array and real-time RT-PCR analyses revealed that not only the ECF sigma factors but also TCSs have a major role in sensing antimicrobial peptides. Most importantly, LL-37 induced the genes of three ABC-type transporters; *yxdLM* was induced strongly (about 700-fold) and its close homologues *yvcRS* and *bceAB* were induced moderately (about sixfold). All these ABC-transporter genes are regulated by TCSs. The TCSs are encoded by genes in the immediate upstream regions of the ABC transporter genes, as evidenced by the lack/decrease of the induction in TCS mutants (Mascher *et al.*, 2003; Ohki *et al.*, 2003b; this study) or demonstrated by primer extension and DNase protection experiments (Pascale *et al.*, 2004). In this study, the results suggested some low-level cross-talk between the three TCSs.

It has been shown that the expression of *bceAB* is induced more than 200-fold by bacitracin via BceRS TCS-mediated signalling (Mascher *et al.*, 2003) and that the BceAB ABC transporter has a role in bacitracin resistance (Ohki *et al.*, 2003b). In contrast, the YxdLM and YvcRS ABC transporters are of unknown function. These TCSs may be involved in sensing conditions inside the cell membrane (Mascher *et al.*, 2003).

This study demonstrates for the first time an activator of the YxdJK TCS: LL-37. Our results also indicate that Triton X-100 and PLL do not activate YxdJK, suggesting distinctly different modes of action for LL-37 and detergents/detergent-like molecules, in contrast to what has been claimed (Oren *et al.*, 1999). LL-37 is most probably a pore-forming peptide (Henzler Wildman *et al.*, 2003), and the penetration of this amphipathic molecule into the membrane is probably crucial for the activation of the YxdJK TCS. The very short extracytoplasmic loop of the YxdK sensor is consistent with the conclusion that YxdJK senses signals inside the membrane, possibly by direct interaction with LL-37, and not signals on the membrane surface or cell wall, or membrane disturbance as such.

PG-1 did not activate YxdJK either, being a pore-forming peptide (Yang *et al.*, 2000), suggesting that the pore formation may not be required for the activation of YxdJK. It may be essential to YxdJK activation that a peptide interacts directly and appropriately with the YxdK sensor in the membrane. In contrast, the LiaRS (YvqCE) TCS, which regulates *liaIHG* expression (Mascher *et al.*, 2004), was strongly activated by both peptides (LL-37 and PG-1) as well as by Triton X-100. Several other stress treatments, such as alkaline shock (Wiegert *et al.*, 2001), vancomycin (Cao *et al.*, 2002b) and secretion stress (H.-L. Hyyryläinen and others, unpublished results) also activate LiaRS (YvqCE). This further confirms that YxdJK senses a narrow range of signals or peptides, while LiaRS broadly senses various stress conditions. Interestingly, however, PLL did not activate LiaRS. These peptide ligands of known structure with differences in their specificity give an excellent future opportunity to study the structure–function relationships of these TCSs.

In addition to *yxdLM* and *liaIH* (*yvqIH*), some other genes of unknown function were also strongly induced by cationic antimicrobial peptides (DNA array). These included *yrhH*, which was induced by both LL-37 and PLL. The *yrhH* gene encodes a putative methyltransferase. *yhcG* was strongly upregulated by LL-37 (14·7-fold) and enhanced expression levels of several other genes of the *yhc* operon were also observed. *yhcG* encodes an ABC-transporter ATP-binding protein. The *yhcH* gene, which was induced 4·6-fold, also encodes a putative (second) ABC-transporter

ATP-binding protein, and the *yhcI* gene, which was induced 5·1-fold, encodes a putative ABC-transporter permease homologous with bacitracin permeases. The putative roles of these ABC transporters in the removal of LL-37 from cells should be studied in the future.

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II

The density of negative charge in the cell wall influences two-component signal transduction in *Bacillus subtilis*

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The Dlt system modulates the density of negative charge in the cell wall of Gram-positive bacteria by substituting anionic polymers (wall and lipoteichoic acids) with D-alanine. The *htrA* and *htrB* genes, regulated by the CssRS two-component system (TCS) and encoding membrane-associated protein quality control proteases, were expressed at strongly decreased levels in a mutant with defective Dlt (*dltD*::miniTn*10*) as compared to the *dlt*$^+$ wild-type strain under a secretion stress condition (hypersecretion of AmyQ α-amylase). The level of HtrA protein in the extracellular proteome of the *dltD* mutant was decreased consistently. Expression from the promoter of the *liaIHGFSR* (*yvqIHGFEC*) operon (P_{liaI}) is dependent on the LiaRS TCS. The Dlt defect increased the expression from P_{liaI} under two stress conditions, AmyQ hypersecretion and treatment with a cationic antimicrobial peptide (LL-37), but decreased the expression in vancomycin-treated cells. Furthermore, Dlt inactivation enhanced the expression of the YxdJK-regulated *yxdL* gene in LL-37-treated cells. The increased net negative charge of the cell wall seems to cause varied and opposite effects on the expression of CssRS-, LiaRS- and YxdJK-regulated genes under different stress conditions. The results suggest that TCSs which sense misfolded proteins or peptides are modulated by the density of negative charge in the cell wall. The density of negative charge on the outer surface of the cell membrane did not have a similar effect on TCSs.

INTRODUCTION

Two-component signal transduction is involved in controlling the quality of exported proteins in bacteria (Raivio & Silhavy, 1999; Sarvas *et al.*, 2004). In *Bacillus subtilis*, the CssRS two-component system (TCS), the *B. subtilis* functional orthologue of the *Escherichia coli* CpxRA TCS (Connolly *et al.*, 1997; Danese & Silhavy, 1997; Missiakas & Raina, 1997; Pogliano *et al.*, 1997), responds to the accumulation of misfolded proteins at the membrane–cell wall interface (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). CssRS-mediated signalling induces the *htrA* and *htrB* genes, which encode serine-type surface proteases involved in quality control. The expression of these quality-control proteases is strictly dependent on CssRS (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). Severe secretion stress and consequent accumulation of misfolded proteins at the membrane–cell wall interface also induces several other genes in *B. subtilis* (Hyyryläinen *et al.*, 2005), including the *liaIHGFSR* operon, the expression of which is also induced by several other stress conditions such as alkaline shock, detergents and cationic antimicrobial peptides (Wiegert *et al.*, 2001; Mascher *et al.*, 2003; Pietiäinen *et al.*, 2005). The last two genes of the *lia* operon encode a TCS, LiaRS, which regulates expression from the P_{liaI} promoter in front of the *liaI* gene (Mascher *et al.*, 2004). The *liaF* gene encodes a membrane-bound

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Abbreviations: CAMP, cationic antimicrobial peptide; QRT-PCR, quantitative real-time RT-PCR; TCS, two-component system.

negative regulator of the LiaRS TCS (Jordan *et al.*, 2006). LiaH is similar to *E. coli* phage shock protein A (PspA). It negatively modulates (weakly) the activity of LiaR-dependent promoters (Jordan *et al.*, 2006), whereas the functional roles of LiaI and LiaG are less well known. In addition to P_{liaI}, there is a weak promoter in the immediate upstream region of *liaG*, P_{liaG}, from which the *liaGFSR* genes are expressed in unstressed cells (Jordan *et al.*, 2006).

PrsA3 is a mutant variant of the PrsA lipoprotein of *B. subtilis*, a membrane-bound peptidyl-prolyl *cis*/*trans*-isomerase (Hyyryläinen *et al.*, 2000; Kontinen *et al.*, 1991). We have shown previously that PrsA3 is highly sensitive to cell-associated proteolytic degradation and consequently its cellular level is decreased to only about 10 % of the wild-type level (Hyyryläinen *et al.*, 2000). We have observed that PrsA3 was stabilized at the membrane–cell wall interface in a similar manner in both *cssS* and *dltD* mutants (Hyyryläinen *et al.*, 2000, 2001). Mutations in *dlt* increase the density of negative charge in the cell wall matrix. In addition to PrsA3, some other mutant and heterologous proteins are also stabilized and their secretion improved when Dlt is inactivated (Craynest *et al.*, 2003; Hyyryläinen *et al.*, 2000; Thwaite *et al.*, 2002; Vitikainen *et al.*, 2005).

Four of the five proteins encoded by the *dlt* operon (DltA, B, C and D) comprise the Dlt system (Perego *et al.*, 1995). Dlt modifies both lipoteichoic and wall teichoic acids with D-alanine and thereby decreases the density of negative charge in the cell wall. The *dlt* operon is under the transcriptional control of σ^X, a sigma factor with an extracytoplasmic function, which belongs to the *Bacillus* cell envelope stress response network (Cao & Helmann, 2004) The improved stability of PrsA3 and other abnormal proteins in *dlt* mutants raises the question of whether the altered charge of the wall affects post-translocational folding of these proteins and thereby stabilizes them, as suggested previously (Hyyryläinen *et al.*, 2000). Alternatively, Dlt could modulate the quality control of exported proteins. The increased net negative charge of the wall in *dlt* mutants may increase the binding of divalent cations to the wall and the bound cations may improve post-translocational folding of exported proteins (Hyyryläinen *et al.*, 2000). Alternatively, the inactivation of Dlt could 'desensitize' the CssRS TCS to misfolded proteins, decrease CssRS-mediated signal transduction, and consequently decrease the expression of the HtrA and HtrB quality control proteases. Indeed, we could demonstrate in this study that in the absence of Dlt, *htrA* and *htrB* were expressed at significantly lower levels than in its presence. It was found that Dlt modulates TCS activity in a manner that is dependent on TCS and the type of inducer/stress.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in either Luria–Bertani broth (LB; 1 % tryptone, 0.5 % yeast extract, 1 % NaCl) or modified 2× LB broth (2× LB_5; 2 % tryptone, 1 % yeast extract, 1 % NaCl) supplemented with appropriate antibiotics, unless otherwise indicated. The final concentrations of the antibiotics were 10 μg kanamycin ml^{-1}, 5 μg chloramphenicol ml^{-1} and 1 μg erythromycin ml^{-1}. For gene expression measurements, bacteria were grown in the absence of antibiotics to avoid putative antibiotic effects on gene expression. Expression from the P_{spac} and P_{xyn} promoters was induced by adding 1 mM IPTG and 0.2 % xylose, respectively, to the growth medium.

Two plasmids, pKTH10 and pKTH3339, were used to cause secretion stress. The pKTH10 plasmid is a derivative of pUB110 with an insert containing the *amyQ* gene of *Bacillus amyloliquefaciens* (Palva *et al.*, 1982). In pKTH3339 (a derivative of pSX50; Hastrup & Jacobs, 1990), *amyQ* has been placed under P_{xyn} control (Vitikainen *et al.*, 2001). Strains harbouring these plasmids secrete AmyQ at high levels into the extracellular medium. The LL-37 antimicrobial peptide (purchased from Dr Ale Närvänen, University of Kuopio, Finland, or from EzBiolab) and vancomycin were added in the early exponential growth phase (~60 Klett units, OD_{560}=0.6) to cause cell envelope stress, and were used at the subinhibitory concentrations of 4 or 6 μg ml^{-1} and 2 μg ml^{-1}, respectively. Triton X-100 was added at the density of 100 Klett units (to cause stress) and used at a concentration of 0.005 %.

Transcription analysis by quantitative real-time RT-PCR (QRT-PCR). Total cellular RNA was isolated for QRT-PCR as described previously (Pietiäinen *et al.*, 2005) or as follows. RNA was extracted by using the Roche High Pure RNA isolation kit according to the manufacturer's instructions. Bacteria from 2 ml of an exponential-phase culture were resuspended in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0, 1 mg lysozyme ml^{-1}) and incubated at 37 °C for 10 min. The subsequent steps were performed as described by the kit manufacturer. If cells were treated with LL-37, vancomycin or Triton X-100 to cause stress, the treatments were continued for 10 or 20 min, followed by harvesting of the cells and extraction of the RNA. Secretion stress was caused by overexpressing the α-amylase of *B. amyloliquefaciens* (AmyQ) from the pKTH3339 plasmid (Hyyryläinen *et al.*, 2001). P_{xyn}-*amyQ* in pKTH3339 was induced with 0.2 % xylose at the density of 60 Klett units for 30 min, followed by RNA extraction. cDNA synthesis was carried out by using the high capacity cDNA archive kit (Applied Biosystems) with an additional step of DNase I treatment. Each reaction mixture consisted of 5 μl 10× buffer, 2 μl 25 mM dNTP, 5 μl hexamer, 11 μl 25 mM $MgCl_2$, 5 μl 20 U DNase (Roche) and 1 μg RNA in 50 μl total volume. The mixture was divided into two equal portions and 1.3 μl reverse transcriptase (MultiScribe) was added to one. The cDNA synthesis reaction was carried out in a thermal cycler with the following steps: (i) DNase I treatment at 37 °C for 30 min, (ii) inactivation of DNase I by incubating at 75 °C for 5 min, (iii) cooling to 4 °C, (iv) addition of reverse transcriptase, (v) cDNA synthesis at 25 °C for 10 min, and (vi) at 37 °C for 120 min. QRT-PCR was carried out as described previously (Pietiäinen *et al.*, 2005) using the SYBR Green PCR master mix (Applied Biosystems) and a 7500 real-time PCR system (Applied Biosystems). The cDNA values were normalized with the value of *gyrA* (Pietiäinen *et al.*, 2005). Sequences of the QRT-PCR primers for the genes studied are shown in Table 2.

pMUTIN constructions and β-galactosidase assay. The pMUTIN2 (Vagner *et al.*, 1998) inserts in the *liaI* and *mprF* (*yfiW* and *yfiX*) genes constructed in the *Bacillus subtilis* Functional Analysis (BSFA) program (Kobayashi *et al.*, 2003) were used to study expression of these genes. Due to a sequencing error (an extra cytidine nucleotide) at position 916935 of the *B. subtilis* genome sequence, the *mprF* gene has been misidentified as two different genes, *yfiW* and *yfiX* (Staubitz & Peschel, 2002). The pMUTIN2 insertion sites in the *yfiW*::pMUTIN2 and *yfiX*::pMUTIN2 mutants are located at 248 and 977 (1 is the first nucleotide of the initiation

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and characteristics	Reference
Strains		
IH6531	*trpC2 glyB133 hisA1* pKTH10	Leskelä *et al.* (1999)
IH6538	*trpC2 glyB133 hisA1*	Leskelä *et al.* (1999)
IH7144	*prsA3 glyB133 hisA1* pKTH10	Hyyryläinen *et al.* (2000)
IH7171	*prsA3 glyB133 hisA1*	Hyyryläinen *et al.* (2001)
IH7375	*dltD*::miniTn*10 trpC2 glyB133 hisA1*	Vitikainen *et al.* (2005)
IH7378	*dltD*::miniTn*10 trpC2 glyB133 hisA1* pKTH10	This study
IH7558	*prsA3 glyB133 hisA1* pKTH3339	Hyyryläinen *et al.* (2001)
IH7612	*htrA*::pMUTIN2 *trpC2 glyB133 hisA1* pKTH10	This study
IH7663	*yhcT*::pMUTIN2 *prsA3 glyB133 hisA1* pKTH3339	Hyyryläinen *et al.* (2001)
IH7988	*htrB*::pMUTIN4 *trpC2 glyB133 hisA1* pKTH10	This study
IH8112	*liaR*::pMUTIN2 *prsA3 glyB133 hisA1* pKTH3339	This study
IH8122*	*trpC2 hisA1 glyB133* pKTH10; parental strain	Leskelä *et al.* (1999)
IH8123*	*trpC2 hisA1 glyB133*; parental strain	Leskelä *et al.* (1999)
IH8149	*htrA*::pMUTIN2 *dltD*::miniTn*10 trpC2 glyB133 hisA1* pKTH10	This study
IH8205	*liaI*::pMUTIN2 *dltD*::miniTn*10 trpC2 glyB133 hisA1* pKTH10	This study
IH8207	*liaI*::pMUTIN2 *trpC2 glyB133 hisA1* pKTH10	This study
IH8219	*liaI*::pMUTIN2 *trpC2 glyB133 hisA1*	This study
IH8220	*liaI*::pMUTIN2 *dltD*::miniTn*10 trpC2 glyB133 hisA1*	This study
IH8268	*htrB*::pMUTIN4 *dltD*::miniTn*10 trpC2 glyB133 hisA1* pKTH10	This study
IH8394	*mprF*(*yfiX*)::pMUTIN2 *trpC2 glyB133 hisA1*	This study
IH8396	*mprF*(*yfiX*)::pMUTIN2 *trpC2 glyB133 hisA1* pKTH10	This study
IH8429	*psd1*::*neo trpC2 glyB133 hisA1*	This study
IH8430	*mprF*(*yfiW*)::pMUTIN2 *trpC2 glyB133 hisA1*	This study
IH8434	*psd1*::*neo prsA3 glyB133 hisA1* pKTH3339	This study
Plasmids		
pKTH10	P_{amyQ}-*amyQ*; Km^r	Palva *et al.* (1982)
pKTH3339	P_{xyn}-*amyQ*; Cm^r	Vitikainen *et al.* (2001)

*IH8122 and IH8123 are reisolations of IH6531 and IH6538, respectively (Leskelä *et al.* 1999).

codon) in the 2568 nt *mprF* gene, respectively. The *htrA*::pMUTIN2 and *htrB*::pMUTIN4 constructs used in this study have been described previously (Hyyryläinen *et al.*, 2001; Darmon *et al.*, 2002). β-Galactosidase activity was determined with ONPG (Merck) substrate as described previously (Msadek *et al.*, 1990) and the activity was expressed in Miller units per milligram total protein.

2D gel electrophoresis analysis. To prepare extracellular protein extracts, *B. subtilis* strains were grown at 37 °C under vigorous agitation in LB medium. After 1 h of post-exponential growth, cells were separated from the growth medium by centrifugation. The secreted proteins in the growth medium were precipitated with 10 % TCA and prepared for 2D PAGE, as described previously (Antelmann *et al.*, 2001). IEF was performed as described previously (Buttner *et al.*, 2001) using commercially available IPG Strips (Amersham Biosciences) in the pH range 3–10 for extracellular proteins. Separation in the second dimension was performed using 12 % polyacrylamide gels. Analytical gels were loaded with 200 μg protein. Gels were stained with either silver nitrate (O'Connell & Stults, 1997) or SYPRO Ruby protein gel stain (Molecular Probes) according to the

Table 2. Specific primer pairs for QRT-PCR

Gene	Forward primer	Reverse primer
gyrA	5′-GATTATTAACCTGCTGGAGGTAGAAAA-3′	5′-AGGTAAAGCTCCGCATTGAATTC-3′
liaI (*yvqI*)	5′-CATCTGCTCACTTCCGTTTGTC-3′	5′-TTCCAGCCGTAATACACCATAGC-3′
liaH (*yvqH*)	5′-TCCAAAGGTGATGCTGAATCAA-3′	5′-GTTTCACAATTGTTTGCTTTGCTT-3′
liaG (*yvqG*)	5′-GCCGGGAATGACCATCTTG-3′	5′-CGTCCGTCACGCTCGTATTT-3′
liaF (*yvqF*)	5′-CCGACATGCGCAGCTTTT-3′	5′-TCAGGTCAAACGGCTGCTT-3′
liaS (*yvqE*)	5′-GATCAGGATGGTCGAGCATATG-3′	5′-GCAGCGCCCTCATCTCATT-3′
liaR (*yvqC*)	5′-CGCGCCGCAAGCAA-3′	5′-CGCAGCCTGGATAATACTTTTCC-3′
yxdL	5′-GGTCATTCATAAGCCGTCACTCA-3′	5′-TTGTCGCTTTGGAATCAAGGT-3′

manufacturer's instructions. Proteins were identified using MALDI-TOF MS, as described previously (Antelmann *et al.*, 2002). Quantitative image analysis was performed by using the false-colour image analysis and quantification tool of the DECODON Delta 2D software (http://www.decodon.com).

RESULTS

Induction of *htrA* and *htrB* by secretion stress is abolished in the absence of D-alanylated teichoic acids

In order to find out whether the increased net negative charge of the cell wall in *dlt* mutants causes down-regulation of expression of the *htrA* and *htrB* genes and thereby stabilization of the PrsA3 protein, a *lacZ* reporter was placed under the control of the protease gene promoters and *β*-galactosidase activity was measured. The *lacZ* reporter was placed under the transcriptional control of the *htrA* and *htrB* genes by inserting pMUTIN plasmids (Vagner *et al.*, 1998) into the protease genes. The strains expressed AmyQ α-amylase from the pKTH10 plasmid and secreted it at a high level (hypersecretion). This caused secretion stress, activated signal transduction via the CssRS TCS and induced the CssRS-regulated P_{htrA} and P_{htrB} promoters (Hyyryläinen *et al.*, 2001).

In the wild-type strain, AmyQ hypersecretion resulted in about 10-fold induction of P_{htrA} expression (Fig. 1a), consistent with previously published results (Hyyryläinen *et al.*, 2001). Secretion stress caused a similar induction of expression from the P_{htrB} promoter (Fig. 1b). In contrast, hardly any induction of P_{htrA} and P_{htrB} was observed in the *dltD* mutant (Fig. 1a, b; representative results of two experiments are shown in each panel). These results suggest that the D-alanylation of teichoic acids modulates signal transduction via CssRS.

Extracellular proteomes of the *dltD* mutant and the wild-type strain were compared using 2D gel electrophoresis. False-colour images of the proteomes (Fig. 2; representative results of two experiments are shown) revealed that the majority of the protein spots were yellow, suggesting that most extracellular proteins were secreted at similar levels from the two strains. A significant fraction of the proteins in the extracellular proteome were cytoplasmic proteins, probably released into the culture medium by cell lysis (Tjalsma *et al.*, 2004). The cytoplasmic proteins included CitH, Eno, SodA and PdhD. The false-colour images of the spots for these proteins suggested that the two strains were not significantly different in their degree of cell lysis (Fig. 2).

The normally cell membrane/wall-associated HtrA protease is also present in the extracellular proteome in abundant amounts (Tjalsma *et al.*, 2004). A prominent finding in this study was that HtrA accumulated at a significantly lower level in the culture medium of the *dltD* mutant than in that of the wild-type strain (Fig. 2). This is in good agreement with the decreased transcription of the *htrA* gene in the *dltD* mutant. Consistent with the decreased amount of HtrA in the proteome of the *dltD* mutant, we also detected decreased secretion of YqxI, which is probably stabilized post-transcriptionally by the chaperone-like activity of HtrA in response to secretion stress (Antelmann *et al.*, 2003). The extracellular proteome analysis further suggested that some other proteins were also present at decreased levels in the culture medium of the *dltD* mutant, including the PbpX penicillin-binding protein, and YrpD and YxkC, whose functions are unknown.

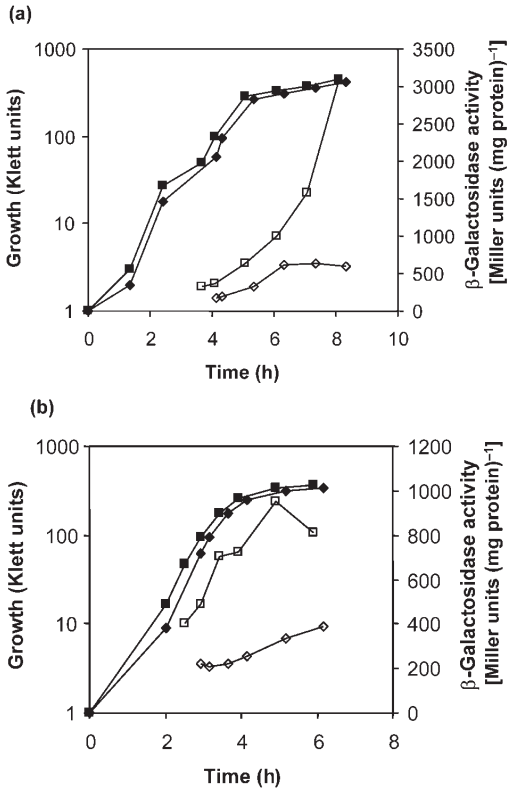

Fig. 1. The effect of *dltD*::miniTn*10* on the expression of *htrA* and *htrB*. (a) Strains IH8149 (*dltD*::miniTn*10* *htrA*::pMUTIN2 pKTH10) and IH7612 (*dlt*$^+$ *htrA*::pMUTIN2 pKTH10) were cultivated in 2× LB_5, and *β*-galactosidase activities of cell samples taken at the indicated time points during growth were determined. The strains secreted AmyQ α-amylase at high levels due to the presence of the pKTH10 plasmid. Squares, *dlt*$^+$; diamonds, *dltD*::miniTn*10*; filled symbols, growth; open symbols, *β*-galactosidase activity. (b) Experiment performed with strains IH7988 (*dlt*$^+$ *htrB*::pMUTIN4 pKTH10) and IH8268 (*dltD*::miniTn*10* *htrB*::pMUTIN4 pKTH10) as in (a).

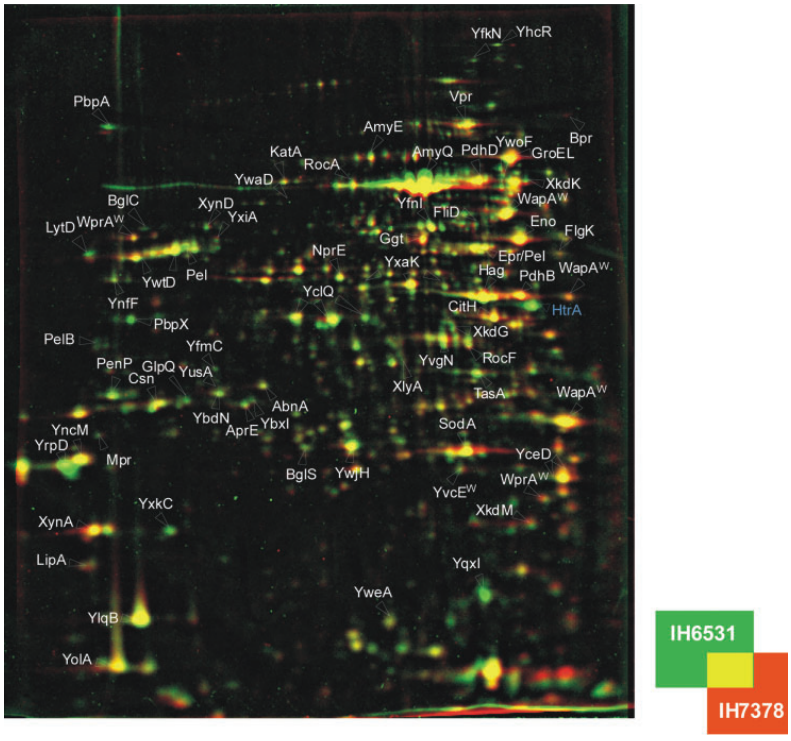

Fig. 2. Comparison of extracellular proteomes of the *dltD*::miniTn*10* mutant and its wild-type (*dlt*$^+$) parent. Superimposed false-colour images of extracellular proteomes of IH6531 (*dlt*$^+$ pKTH10) and IH7378 (*dltD*::miniTn*10* pKTH10) are shown. Extracellular proteins were TCA-precipitated from the culture medium of late-exponential-phase cultures and separated by 2D gel electrophoresis. The colour code of the spots indicates the relative abundance of the proteins in the extracellular proteomes of the two strains. The spot for HtrA protease is indicated by blue type.

Induction of expression from the LiaRS-regulated P_{liaI} promoter in secretion stress and stress caused by cationic antimicrobial peptides is dependent on the cell wall charge

The *liaIHGFSR* gene cluster is strongly induced in an unspecific manner by antibiotics that interfere with the lipid II cycle, membrane-active cationic antimicrobial peptides (CAMPs) such as LL-37, detergents such as Triton X-100 (Mascher *et al.*, 2004; Pietiäinen *et al.*, 2005) and severe secretion stress (AmyQ secretion at a high level and a defect in the PrsA petidyl-prolyl *cis*/*trans* isomerase; Hyyryläinen *et al.*, 2005). Expression from the *liaI* promoter (P_{liaI}) is strictly dependent on the LiaRS TCS, encoded by the *liaR* and *liaS* genes in the same gene cluster (Mascher *et al.*, 2003). Severe secretion stress (*amyQ* expressed from pKTH3339 and impaired post-translocational folding due to the *prsA3* mutation) caused no induction of *liaI* expression in a knockout mutant of *liaR*, whereas a 77-fold induction (QRT-PCR measurement) was seen in the wild-type. A similar result was obtained with LL-37: *liaI* was induced 55-fold in wild-type cells and not at all in *liaR* mutant cells. These results are similar to the LiaRS-dependent induction of *liaIH* in cells treated with bacitracin (Mascher *et al.*, 2003).

In order to find out whether the Dlt system, and consequently the density of negative charge in the cell wall, modulates stress responses mediated by two-component sensory systems other than CssRS, the effect of *dltD*::miniTn*10* on the expression from the LiaRS-regulated P_{liaI} promoter was determined. The pMUTIN2 plasmid (Vagner *et al.*, 1998) was inserted into the *liaI* gene. The plasmid insert probably inactivated *liaI*, placed a *lacZ* reporter under the control of P_{liaI} and placed the downstream genes of the *lia* operon under the control of the IPTG-inducible P_{spac} promoter. The P_{liaI} promoter was induced by either subjecting the cells to AmyQ hypersecretion or treating them with LL-37.

The effect of the P_{spac} induction on the expression of the downstream genes, *liaHGFSR*, was determined in the

absence and presence of IPTG by using QRT-PCR. In the absence of IPTG, they were expressed at low levels (Table 3). However, the expression levels of *liaGFSR* were clearly higher than that of *liaH* (>10-fold higher levels of cDNA), consistent with the presence of the weak P_{liaG} promoter in the upstream region of *liaG* (Jordan *et al.*, 2006). When P_{spac} was induced with 1 mM IPTG, all five downstream genes were strongly induced. In the case of *liaH*, the induction was about 500-fold as compared to the level in the absence of IPTG. The expression levels were similar in the *dlt*$^+$ (wild-type) strain (IH8207) and the *dltD*::miniTn*10* mutant (IH8205).

P_{liaI} expression was first determined in the absence of IPTG (the P_{spac}-*liaHGFSR* genes expressed at low levels) by measuring *β*-galactosidase activity. In wild-type cells secreting AmyQ, P_{liaI} was induced only slightly (Fig. 3a). This is consistent with our previous DNA macroarray results (Hyyryläinen *et al.*, 2005) showing that the *lia* operon is not significantly induced by mere AmyQ hypersecretion, but it is induced if there is also a mutation in the gene encoding the post-translocational folding factor PrsA. In the present study, about fivefold higher P_{liaI} expression levels (LacZ measurement) were observed in the *dltD*::miniTn*10* mutant than in the wild-type strain (Fig. 3a). In the presence of IPTG and thus the downstream P_{spac}-*liaHGFSR* genes expressed at higher levels than in its absence, the results were distinctly different. In the wild-type strain, the overall P_{liaI} expression levels were similar to those in the absence of IPTG (Fig. 3a, b). However, the *dltD*::miniTn*10* mutation conferred no expression-enhancing effect on P_{liaI} (Fig. 3b).

When cells were treated with LL-37, the expression from P_{liaI} was clearly upregulated (up to ~10-fold) in the *dltD*::miniTn*10* mutant under both conditions (with or without IPTG; Fig. 4a, b). The P_{liaI} expression returned to the low initial level in about 4 h from the addition of LL-37, possibly due to elimination of the peptide in the cells. In the presence of IPTG, the overall P_{liaI} expression levels were lower (about fourfold reduced) as compared to the expression levels in the absence of IPTG, and the induction was also more transient (Fig. 4a, b). In the wild-type strain (in the absence of IPTG) only a small induction peak was observed (Fig. 4a). These results are similar to and consistent with those obtained for secretion stress.

Table 3. Expression levels of the *lia* genes downstream of the *liaI*::pMUTIN2 insertion site and under the control of the P_{spac} promoter

Gene	Expression levels of P_{spac}-*liaHGFSR**			
	dlt$^+$		*dltD*::miniTn*10*	
	−IPTG	+IPTG	−IPTG	+IPTG
liaH	0.002	1.065	0.004	2.396
liaG	0.081	3.008	0.155	2.082
liaF	0.038	0.526	0.041	0.294
liaS	0.173	2.014	0.305	1.758
liaR	0.131	1.055	0.190	0.784

*Strains (IH8205 and IH8207) were grown in either the absence or the presence of IPTG to a cell density of 60 Klett units, and P_{spac}-*liaHGFSR* gene expression levels were determined by QRT-PCR. Values are ng DNA.

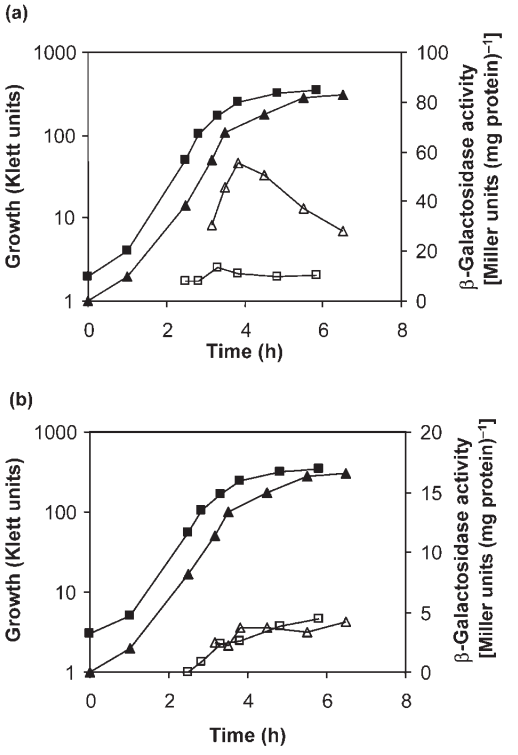

Fig. 3. Induction of P_{liaI} in the *dltD*::miniTn*10* mutant by secretion stress. (a) Strains IH8205 (*dltD*::miniTn*10* *liaI*::pMUTIN2 pKTH10) and IH8207 (*dlt*$^+$ *liaI*::pMUTIN2 pKTH10) secreting AmyQ *α*-amylase at high levels were cultivated in 2× LB_5, and *β*-galactosidase activities of cell samples taken at the indicated time points during growth were determined. The *lia* genes downstream of *liaI* (P_{spac}-*liaHGFSR*) were not induced. Squares, *dlt*$^+$; triangles, *dltD*::miniTn*10*; filled symbols, growth; open symbols, *β*-galactosidase activity. (b) As in (a), but P_{spac}-*liaHGFSR* was induced with 1 mM IPTG.

The pMUTIN2 insertion in the strains of the above experiments probably inactivated the *liaI* gene. In order to ensure that *liaI* disruption did not modulate the response patterns of P_{liaI} under stress, we also determined the expression levels of the *lia* genes in the strain pair IH8123 (*dlt*$^+$) and IH7375 (*dltD*::miniTn*10*), which do not contain the pMUTIN2 insertion in *liaI*, by using QRT-PCR. Cells of

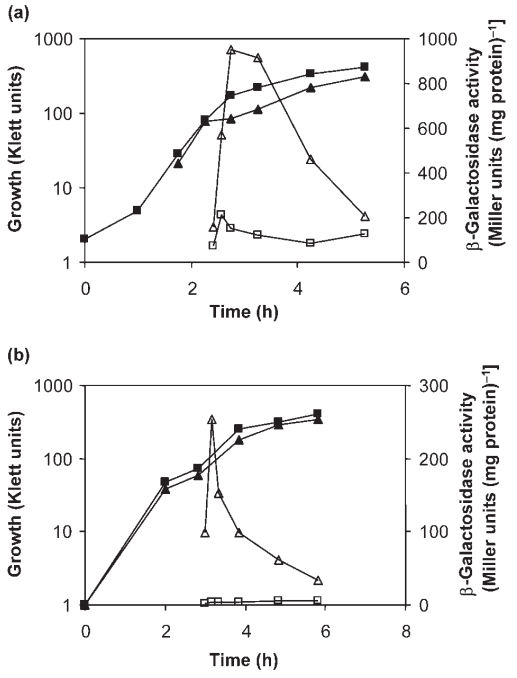

Fig. 4. Induction of P_{liaI} in the *dltD*::miniTn*10* mutant by LL-37. (a) Strains IH8220 (*dltD*::miniTn*10* *liaI*::pMUTIN2) and IH8219 (*dlt*$^+$ *liaI*::pMUTIN2) were cultivated in 2× LB_5 and treated with LL-37 at a cell density of 60 Klett units for 20 min, followed by determination of *β*-galactosidase activities of cell samples taken at the indicated time points during growth. The *lia* genes downstream of *liaI* (P_{spac}-*liaHGFSR*) were not induced. Squares, *dlt*$^+$; triangles, *dltD*::miniTn*10*; filled symbols, growth; open symbols, *β*-galactosidase activity. (b) As in (a), but P_{spac}-*liaHGFSR* was induced with 1 mM IPTG.

these strains were treated with LL-37 for 10 or 20 min, and *lia* gene expression levels were determined and compared with those in non-treated cells (Table 4). The fold induction values were significantly higher than those obtained with the *β*-galactosidase activity measurements due to differences in the sensitivities of the methods. However, consistent with the *β*-galactosidase activities (P_{spac} not induced), LL-37 caused a much stronger induction of P_{liaI} in the *dltD*::miniTn*10* mutant than in the wild-type. This stronger induction was evident in the fold induction values of all six *lia* genes at both time points. The *liaI* and *liaH* genes were induced in both strains more strongly than the other *lia* genes, consistent with the presence of a potential transcription termination loop after *liaH* (Jordan *et al.*, 2006).

Induction of P_{liaI} by vancomycin or Triton X-100 is not enhanced in the *dltD*::miniTn*10* mutant

We also studied whether the density of negative charge in the cell wall modulates the response of LiaRS to another cationic-type inducer, the glycopeptide antibiotic vancomycin. Furthermore, we used a non-ionic inducer of LiaRS, the detergent Triton X-100, to find out if the Dlt effect is restricted to cationic inducers such as peptides or if the response to a non-ionic inducer is also affected by the Dlt system. Cells of the *dltD*::miniTn*10* mutant and the wild-type strain were treated with vancomycin (2 μg ml^{-1}) or 0.005 % Triton X-100 for 10 or 20 min and P_{liaI} expression was determined using QRT-PCR.

In the wild-type strain, vancomycin enhanced P_{liaI} expression (QRT-PCR measurements) hundredsfold as compared to the expression level in non-treated cells (Table 5), consistent with the published results (Mascher *et al.*, 2004) and showing that vancomycin is a strong inducer of the LiaRS TCS. In the *dltD*::miniTn*10* mutant, the response to vancomycin was significantly lower (at least sixfold reduced expression of P_{liaI}), in contrast to the stronger response in

Table 4. Induction of the expression of the *lia* genes in the *dltD*::miniTn*10* mutant and the *dlt*$^+$ wild-type strain by LL-37

Gene	Fold induction for strain:*			
	IH8123 (*dlt*$^+$)		IH7375 (*dltD*::miniTn*10*)	
	10 min	20 min	10 min	20 min
liaI	19.7 (20.6, 18.7)	13.4 (12.0, 14.8)	379.1 (532.3, 225.9)	318.8 (123.0, 514.5)
liaH	17.9 (14.9, 20.8)	15.3 (12.9, 17.6)	300.4 (391.1, 209.7)	479.7 (140.5, 818.9)
liaG	1.7 (1.4, 2.0)	1.6 (1.6, 1.6)	29.8 (42.9, 16.7)	7.3 (5.6, 9.0)
liaF	2.4 (2.0, 2.7)	1.5 (1.8, 1.1)	14.9 (10.1, 19.7)	6.7 (4.1, 9.2)
liaS	1.2 (1.1, 1.3)	1.4 (1.6, 1.2)	20.9 (29.6, 12.1)	2.9 (2.3, 3.4)
liaR	1.3 (1.1, 1.4)	1.0 (1.0, 0.9)	8.3 (3.7, 12.9)	2.0 (2.5, 1.5)

*The ratio of the expression level of the gene in LL-37-treated cells to that in untreated cells, as determined by QRT-PCR. The induction ratios are means from two independent experiments.

the LL-37-treated *dltD*::miniTn*10* mutant cells. The fold induction values were similar at both time points.

In the cells treated with Triton X-100, the induction time-course of P_{liaI} was such that at the 10 min time point the fold induction was about 60, and it increased to about 150 at the 20 min time point (Table 5). There were no significant differences in the induction patterns of the two strains under this stress condition.

Dlt modulates the response of the YxdJK TCS to LL-37

The results of the above experiments suggested that cell wall charge can modulate TCS activity in different ways depending on the TCS and properties of the inducer molecule. YxdJK is a TCS which is activated by LL-37 in a highly specific manner (Pietiäinen *et al.*, 2005). We used YxdJK as the third sensory system to study the Dlt dependence of TCS activation. It was observed that Dlt affects the activity of YxdJK in a similar manner to that of LiaRS, i.e. the activity was enhanced in the *dltD*::miniTn*10* mutant. There was about a threefold higher induction of the YxdJK-regulated *yxdL* gene in the *dltD*::miniTn*10* mutant as compared to the wild-type strain at both the 10 and 20 min time points (Table 5).

Modulating the negative charge of the outer surface of the cell membrane does not affect the activity of TCSs

In a similar manner to the negative charge of the cell wall, the negative charge of the cell membrane surface could also affect the activity of TCSs. We used mutations that decrease positively charged amines in the membrane and thereby increase the negative charge of the membrane surface. The inactivated components were MprF, which modifies membrane lipids with L-lysine (Peschel *et al.*, 2001; Staubitz & Peschel, 2002), and Psd, which encodes phosphatidylserine decarboxylase and is involved in the biosynthesis of phosphatidylethanolamine (Cao & Helmann, 2004; Matsumoto *et al.*, 1998). Both *Staphylococcus aureus* and *B. subtilis* synthesize lysylphosphatidylglycerol, and it has been shown with *S. aureus* that MprF is responsible for the synthesis (Peschel *et al.*, 2001; Staubitz & Peschel, 2002). Two pMUTIN2 insertion mutations of the *B. subtilis mprF* ortholoque, *yfiW*::pMUTIN2 and *yfiX*::pMUTIN2, were used in this study to characterize the putative role of MprF in the activation of LiaRS and YxdJK. The *psd1*::*neo* mutation (Matsumoto *et al.*, 1998) was used to determine the putative role of Psd in the activation of LiaRS, YxdJK and CssRS.

We first determined the sensitivities of the *yfiX*::pMUTIN2 (IH8394) mutant and the wild-type parent (*mprF*$^+$, IH8123) to LL-37. LL-37 completely inhibited the growth of the wild-type *B. subtilis* strain in a liquid culture at a concentration of 12 μg ml^{-1}, delayed the start of growth at 10 μg ml^{-1} and had no effect on growth at 8 μg ml^{-1}. The LL-37 sensitivity of the mutant did not differ from that of the wild-type strain, in contrast to the increased sensitivity of analogous mutants of *S. aureus* to defensins (Peschel *et al.*, 2001). We used a sub-inhibitory concentration of LL-37 (4 or 6 μg ml^{-1}) and determined the induction of P_{liaI} and P_{yxdL} in the *yfiW*::pMUTIN2, *yfiX*::pMUTIN2 and *psd1*::*neo* mutants and the wild-type

Table 5. Effects of the inactivation of *dltD*, *mprF* and *psd* on P_{liaI} and P_{yxdL} expression

Strain	Genotype	Stress	Fold induction*			
			P_{liaI}		P_{yxdL}	
			10 min	20 min	10 min	20 min
IH8123	wt	LL-37	21 (17, 25)	14 (13, 14)	55 (53, 56)	47 (35, 58)
IH7375	*dltD*	LL-37	417 (395, 438)	316 (122, 509)	166 (105, 226)	169 (75, 262)
IH8123	wt	Vancomycin	749 (415)	910 (325)	ND	ND
IH7375	*dltD*	Vancomycin	83 (30)	150 (77)	ND	ND
IH8123	wt	Triton X-100	64 (57, 71)	135 (107, 163)	ND	ND
IH7375	*dltD*	Triton X-100	68 (55, 80)	188 (147, 229)	ND	ND
IH8123	wt	LL-37	18 (16, 20)	12 (8, 16)	55 (53, 56)	47 (35, 58)
IH7375	*mprF* (*yfiX*::pMUTIN2)	LL-37	33 (44, 22)	18 (20, 16)	28 (20, 36)	44 (32, 55)
IH8123	wt	LL-37	1281 (192)†	866 (351)†	323 (181)†	301 (37)†
IH8430	*mprF* (*yfiW*::pMUTIN2)	LL-37	935 (198)†	901 (279)†	258 (78)†	216 (58)†
IH8123	wt	LL-37	614 (137)†	191 (55)†	219 (84)†	135 (25)†
IH8429	*psd1*::*neo*	LL-37	704 (162)†	447 (113)†	287 (66)†	234 (30)†

*The strains were exposed to the indicated stresses, and fold inductions of the *liaI* and *yxdL* genes were determined by QRT-PCR after 10 and 20 min exposure to stress. The fold inductions are means of two or three independent experiments. The fold inductions of the independent experiments (two experiments) or SD (three experiments) are shown in parentheses. ND, Not determined; wt, wild-type.

†In these experiments LL-37 was used at a concentration of 6 μg ml^{-1}, whereas in the others the concentration was lower (4 μg ml^{-1}), explaining the significant difference in the fold induction values.

parental strain by QRT-PCR. The mutations had no significant effects on the induction levels of the two promoters (Table 5). Furthermore, *psd1*::*neo* did not modulate the activation of P_{htrA} by secretion stress [fold inductions: 21.1 (wild-type) versus 19.9 (*psd1*::*neo*)]. These results suggest that the membrane surface charge does not modulate the TCSs.

DISCUSSION

In this study transcription and proteome analyses were used to demonstrate the role of the Dlt D-alanylation system and the net negative charge of the cell wall in two-component signal transduction and gene expression regulation. The transcription measurements and extracellular proteomes of the *dltD* mutant and its wild-type parent revealed that the *htrA* and *htrB* genes were expressed at strongly decreased levels in the *dltD*::miniTn*10* mutant. This finding is consistent with the stabilization of the PrsA3 protein in the *dltD*::miniTn*10* mutant, since the HtrA and HtrB quality-control proteases are partially responsible for the degradation of PrsA3 (Hyyryläinen *et al.*, 2001). It was suggested previously that the increased net negative charge of the cell wall in *dlt* mutants may improve the post-translocational folding of heterologous and mutant proteins that are particularly sensitive to proteolytic degradation (Hyyryläinen *et al.*, 2000). Improved protein folding could decrease stress in the cell envelope and consequently decrease the activity of CssRS and the expression of *htrA* and *htrB*, explaining the findings of the present study and the stabilization of heterologous and mutant proteins in *dlt* mutants. Alternatively, interactions between misfolded secretory proteins and CssS may be different in the absence and presence of D-alanylated teichoic acids due to changes in electrostatic affinity/repulsion forces at the cell wall matrix. Such changes could also affect interactions between lipoteichoic acids and CssS, possibly modulating the activity of the TCS. It is evident, however, that the reason for the improved stability of PrsA3 and some heterologous proteins in *dlt* mutants is probably the decreased levels of the HtrA and HtrB quality control proteases, and consequently decreased degradation. Consistent with the decreased level of HtrA, the level of YqxI (in the extracellular proteome) was also decreased. The secretion and stability of YqxI is probably dependent on HtrA (Antelmann *et al.*, 2003).

The LiaRS system also exhibited a Dlt effect in cells subjected to severe secretion stress or treated with LL-37, but compared to the CssRS system (secretion stress), its direction was the opposite, i.e. a clearly stronger induction of LiaRS, as measured by the expression from P_{liaI}, was observed in the *dltD*::miniTn*10* mutant compared with the wild-type strain (*dlt*$^+$). The enhanced induction was observed with both a P_{liaI}–*lacZ* reporter and by measuring P_{liaI} expression with QRT-PCR. The enhanced LiaRS activity may be due to stronger cell envelope stress in the mutant. Most probably, cationic peptides bind to cell envelopes of *dlt* mutants of Gram-positive bacteria in higher amounts than to those of *dlt*$^+$ strains (Peschel *et al.*, 1999). The increased affinity of *dlt* mutant cell walls to CAMPs subjects the mutant cells to higher stress and increases their sensitivity to CAMPs (Cao & Helmann, 2004; Kristian *et al.*, 2005; Peschel *et al.*, 1999; Poyart *et al.*, 2003).

The glycopeptide antibiotic vancomycin, also a cationic molecule, is one of the strongest inducers of LiaRS (Mascher *et al.*, 2004), but Dlt modulated the response to vancomycin in a different manner than the response to secretion stress or LL-37. It was found that P_{liaI} expression was attenuated in the *dltD* mutant as compared to the *dlt*$^+$ strain. In contrast to the responses to these cationic inducers, the LiaRS response to a non-ionic inducer, Triton X-100, was not affected by Dlt.

These results suggest that Gram-positive bacterial cells can modulate two-component signal transduction and gene expression under stress by modulating the density of negative charge in the wall. The expression of the *dlt* operon is dependent on the extracytoplasmic sigma factor σ^X (Cao & Helmann, 2004), and under some growth conditions probably also on σ^D and the Spo0A and AbrB sporulation proteins (Perego *et al.*, 1995; Cao & Helmann, 2004). Environmental stress conditions, which activate σ^X, modulate the net negative charge of the wall by increasing *dlt* expression and thereby affect the stress response of TCSs. However, the modulation of the stress response may be limited to stress caused by cationic molecules and accumulation of misfolded proteins, and TCSs responding to these stress conditions. Consistent with this conclusion, the YxdJK TCS, which is activated highly specifically by the human cathelicidin LL-37, was also induced in a Dlt-dependent manner. The induction pattern was similar to that of the LiaRS TCS: stronger induction in the *dltD*::miniTn*10* mutant.

The LiaRS and YxdJK TCSs most probably sense a very different type of stress than does CssRS. In addition to our results, such a difference is also suggested by the predicted transmembrane topology of the two-component sensors. The CssS protein has a 137 aa soluble periplasmic region which may have a role in the sensing of misfolded proteins at the membrane–cell wall interface. It is a fairly specific sensor, since membrane-active compounds such as CAMPs or detergents do not activate it. In contrast, LiaS and YxdK do not have a periplasmic domain, suggesting that they are dedicated to sensing intramembrane stress. This may explain why membrane-active CAMPs activate LiaS and YxdK much more strongly than secretion stress. This model may also explain the effect of vancomycin on LiaRS. Since vancomycin strongly activates LiaRS, it probably causes intramembrane stress (e.g. accumulation of cell wall precursors in the membrane), which is alleviated in the absence of D-alanylated teichoic acids.

We also studied whether the degree of lysinylation of the negatively charged phospholipid head groups of the cell

membrane and the proportion of phosphatidylethanolamine in the membrane lipids could modulate the stress response to LL-37 or protein secretion in a similar manner to the D-alanylation of teichoic and lipoteichoic acids. This was not the case. The induction of LiaRS, YxdJK and CssRS was independent of these factors.

The induction of P_{spac}-*liaHGFSR* expression (pMUTIN2 insert at *liaI*) with IPTG repressed P_{liaI} expression. Thorsten Mascher's group (University of Göttingen, Germany) has found that inactivating *liaF* increases expression from the P_{liaI} promoter, indicating that LiaF is a negative regulator of P_{liaI} (Jordan *et al.*, 2006). LiaF is a putative integral membrane protein and may therefore not repress P_{liaI} by directly interacting with the promoter but rather by negatively regulating the activity of LiaRS. LiaH also moderately down-regulates P_{liaI} (Jordan *et al.*, 2006). When the *lia* genes are expressed at low levels (in non-stressed cells), the P_{liaI} promoter is capable of responding strongly if the cells are exposed to stress. Under stress conditions, when the *lia* genes are expressed at high levels, an auto-control mechanism starts to down-regulate P_{liaI} expression. The Lia proteins, which are repressors of P_{liaI}, may have a role in this auto-control, particularly LiaH, since the stoichiometry between LiaH and LiaRS changes dramatically during the activation of the operon.

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III

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Transcriptome analysis of the responses of *Staphylococcus aureus* to antimicrobial peptides and characterization of the roles of *vraDE* and *vraSR* in antimicrobial resistance

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Abstract

Background: Understanding how pathogens respond to antimicrobial peptides, and how this compares to currently available antibiotics, is crucial for optimizing antimicrobial therapy. *Staphylococcus aureus* has several known resistance mechanisms against human cationic antimicrobial peptides (CAMPs). Gene expression changes in *S. aureus* strain Newman exposed to linear CAMPs were analyzed by DNA microarray. Three antimicrobial peptides were used in the analysis, two are derived from frog, temporin L and dermaseptin K4-S4(1-16), and the ovispirin-1 is obtained from sheep.

Results: The peptides induced the VraSR cell-wall regulon and several other genes that are also up-regulated in cells treated with vancomycin and other cell wall-active antibiotics. In addition to this similarity, three genes/operons were particularly strongly induced by the peptides: *vraDE*, SA0205 and SAS016, encoding an ABC transporter, a putative membrane-bound lysostaphin-like peptidase and a small functionally unknown protein, respectively. Ovispirin-1 and dermaseptin K4-S4(1-16), which disrupt lipid bilayers by the carpet mechanism, appeared to be strong inducers of the *vraDE* operon. We show that high level induction by ovispirin-1 is dependent on the amide modification of the peptide C-terminus. This suggests that the amide group has a crucial role in the activation of the Aps (GraRS) sensory system, the regulator of *vraDE*. In contrast, temporin L, which disrupts lipid bilayers by forming pores, revealed a weaker inducer of *vraDE* despite the C-terminal amide modification. Sensitivity testing with CAMPs and other antimicrobials suggested that VraDE is a transporter dedicated to resist bacitracin. We also showed that SA0205 belongs to the VraSR regulon. Furthermore, VraSR was shown to be important for resistance against a wide range of cell wall-active antibiotics and other antimicrobial agents including the amide-modified ovispirin-1, bacitracin, teicoplanin, cefotaxime and 10 other β-lactam antibiotics, chlorpromazine, thioridazine and EGTA.

Conclusion: Defense against different CAMPs involves not only general signaling pathways but also CAMP-specific ones. These results suggest that CAMPs or a mixture of CAMPs could constitute a potential additive to standard antibiotic treatment.

Background

The ubiquitous presence of cationic antimicrobial peptides (CAMPs) in virtually all types of cells and organisms ranging from bacterial cells to humans suggests that CAMPs have important conserved roles as defense weapons. CAMPs contribute to the host defense of microbial invasion on epithelial surfaces by killing engulfed microorganisms in phagocytic cells or modulating inflammatory responses in infections [1-3]. These ancient weapons of host defense are typically amphipathic peptides with a net positive charge at physiological pH and they share well-defined α-helical or β-sheet secondary structures. CAMPs are able to integrate into cell membranes, form membrane-spanning pores and thereby cause lethal cell damage. The positive charge is important for the initial binding of CAMPs to target membranes. There are differences in the way CAMPs interact with membranes and accordingly three different models have been used to define their mode of actions in model membrane systems [4]. In the *barrel-stave mechanism*, peptides integrate into the membrane and form membrane-spanning pores [5]. In the *toroidal-pore mechanism*, CAMPs form membrane-spanning pores together with intercalated lipids [5]. And in the *carpet mechanism*, peptides accumulate on the membrane surface in a carpet-like manner and at a threshold density so that they dissolve the membrane without forming transmembrane channels [6]. However, membrane damage is not the only mechanism whereby CAMPs cause cell death. They may also affect functions of several other cell components and act as metabolic inhibitors of cellular processes including biosynthesis of the cell wall, nucleic-acids and proteins [1,3]. In these cases, the cell death can be the result of multiple inhibitory effects.

Bacteria have evolved mechanisms to combat the harmful effects of CAMPs [3]. On the other hand, the antimicrobial peptide repertoire in host cells may have co-evolved with the evolution of microbial resistance mechanisms. This is exemplified by the skin of the frog *Rana temporaria* in which some temporins act in a synergistic manner to overcome the resistance of Gram-negative bacteria imposed by the lipopolysaccharide [7]. The high resistance of *Staphylococcus aureus* to CAMPs produced by human cells may contribute to epithelial colonization and resistance to destruction by neutrophils. Several mechanisms conferring increased resistance to CAMPs have been identified. The modulation of the density of negative charge in the cell wall by D-alanylation of teichoic acids (Dlt system) or on the outer surface of the cell membrane by L-lysinylation of phosphatidylglycerol (MprF) has been shown to contribute to CAMP resistance in *S. aureus* [8,9] as well as in several other bacteria [10-13]. In Gram-negative bacteria, LPS modifications modulate cell envelope charge and polymyxin susceptibility [14-18]. Many bacteria species produce proteases which cleave antimicrobial peptides, particularly linear ones [3]. *S. aureus* aureolysin and V8 as well as proteases secreted by *Bacillus anthracis* are examples of proteases capable of cleaving the human cathelicidin LL-37 [19,20]. The CAMP resistance mechanisms also include CAMP-binding proteins and CAMP efflux pumps [21-23].

Bacterial sensory systems are capable of recognizing cationic antimicrobial peptides and to respond to their presence by up-regulation of general stress systems as well as more specific CAMP resistance mechanisms. In *Salmonella typhimurium* binding of CAMPs to the PhoQ two-component sensor kinase activates the signal transduction cascade from the sensor to the PhoQ response regulator, resulting consequently in the induction of the PhoPQ-regulated promoters [24]. A complex stress response was observed when *Bacillus subtilis* was exposed to CAMPs, including activation of the SigW and SigM extracytoplasmic sigma factors and the YxdJK and LiaRS two-component systems [25]. In this study, our purpose was to characterize the stress response of *S. aureus* treated with cationic antimicrobial peptides by using whole-genome oligoarrays. The effects of three different α-helical CAMPs on the transcriptome of the *S. aureus* Newman strain were analyzed: temporin L, ovispirin-1 and dermaseptin K4-S4(1-16). These peptides are synthesized as preproproteins (precursors) and the mature microbicidal peptides are formed after proteolytic cleavage of the pre- (signal peptide) and pro-regions of the precursors [26,27]. Ovispirin-1 is a derivative of the cathelicidin SMAP-29 found in sheep [28]. The other two peptides, dermaseptin K4-S4(1-16), which is a truncated derivative of the dermaseptin S4, and temporin L, are both expressed in amphibian skin [29,30]. Temporin L belongs to pore-forming peptides (barrel-stave or toroidal pore mechanism) [30,31] and ovispirin-1 and dermaseptin disrupt lipid bilayers by the carpet mechanism [1]. We used these well-characterized peptides of animal origin as models of cationic antimicrobial peptides and expected that studying their interactions with *S. aureus* would give information that was more generally applicable also to human CAMPs. We aimed to identify the CAMP stimulons and to determine whether the differentially expressed genes play a role in CAMP resistance. Furthermore, we were interested to find out whether there are strong peptide-specific responses or a more general stress response triggered by CAMPs exposition, to explore the mode of action of CAMPs and to improve our understanding of the resistance mechanisms against CAMPs.

Results

The VraSR cell wall regulon, vraDE, SA0205 and SAS016 are strongly induced by cationic antimicrobial peptides

Exponentially growing *S. aureus* cells were treated with C-terminally amidated temporin L (temporin L-NH_2),

ovispirin-1 (ovispirin-1-NH_2) or dermaseptin K4-S4(1-16) (dermaseptin K4-S4(1-16)-NH_2) at sublethal concentrations (see Methods) which slightly inhibited growth but did not stop it. Gene expression changes in the CAMP-treated cells as compared to non-treated cells were analyzed by using whole-genome oligoarrays.

All three peptides upregulated a large number of genes (63-247) and most of them were induced by more than one peptide. Table 1 shows a set of the genes induced by at least one of the peptides at least 3-fold and represents a list of "marker" genes for the CAMP stimulon. The complete list of the induced genes (higher than 2-fold induc-

Table 1: Genes induced by cationic antimicrobial peptides.

Gene ID	Gene name*	Induction with**			Known regulation***	Protein/Similarity
		T	O	D		
SA0011		3.5	2.1			Similar to homoserine-o-acethyltransferase
SA0122	*butA*	3.2	2.1		VCM	Acetoin reductase
SA0205		17.2	11.2	6.2	VCM	Similar to lysostaphin precursor
SA0344	*metE*	4.5	2.3		VCM	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
SA0428		3.6	2.4	2.3		Hypothetical protein
SA0430	*gltB*	4.4	2.5			Glutamate synthase large subunit
SA0480	*ctsR*	4.2	4.2			Transcription repressor of class III stress genes homologue
SA0513		3.3	2.2			Conserved hypothetical protein
SA0591		3.0	3.3	2.1	VCM	Hypothetical protein
SA0677		3.6	2.1		VCM	Similar to choline transport ATP-binding protein
SA0781		3.1	2.1			Similar to 2-nitropropane dioxygenase
SA0817		5.0	2.7			Similar to NADH-dependent flavin oxidoreductase
SA0825	*spsA*	2.6	3.5	2.0	VraSR, VCM	Type-I signal peptidase
SA0835	*clpB*	3.9	4.1		VCM	ClpB chaperone homologue
SA0845	*oppB*	4.3	2.0		VCM	Oligopeptide transport system permease protein
SA0903		2.1	4.1	2.6		Conserved hypothetical protein
SA1164	*dhoM*	5.2	2.8		VCM	Homoserine dehydrogenase
SA1170	*katA*	4.3	2.6			Catalase
SA1216		3.8	2.4			Similar to oligoendopeptidase
SA1219			5.4	4.2		Similar to phosphate ABC transporter
SA1227	*dapA*	8.3	9.6		VCM	Dihydrodipicolinate synthase
SA1254		3.1	3.5		VraSR, VCM	Hypothetical protein
SA1476		4.2	5.3	3.3	VraSR, VCM	Hypothetical protein
SA1517	*citC*	4.9	5.8	3.1	VCM	Isocitrate dehydrogenase
SA1545	*serA*	4.6	2.5		VCM	Similar to soluble hydrogenase 42 kD subunit
SA1549	*htrA*	2.9	3.0	2.0	VraSR, VCM	Similar to serine proteinase Do, heat-shock protein HtrA
SA1599		3.2	2.4			Similar to transaldolase
SA1655	*ecsA*	3.0	3.0			ABC transporter EcsA homologue
SA1659	*prsA*	3.2	4.5	2.9	VraSR, VCM	Peptidyl-prolyl *cis*/*trans* isomerase homologue
SA1701	*vraS*	3.9	5.3	2.5	VraSR, VCM	Two-component sensor histidine kinase
SA1820		3.6	3.0	2.2		Similar to bacteriophage terminase small subunit
SA1836	*groEL*	3.8	4.3	2.2		GroEL protein
SA1862	*leuA*	13.5	3.3	2.1		2-isopropylmalate synthase
SA1990		3.4	2.6	2.2		Conserved hypothetical protein
SA2113		2.2	3.7	2.2	VraSR, VCM	Hypothetical protein
SA2221		2.4	3.2		VraSR, VCM	Hypothetical protein
SA2304	*fbp*	4.8	2.4		VCM	Fructose-bisphosphatase
SA2324		4.9	2.6	2.0		Similar to thioredoxin
SA2343		4.3	4.3	2.7	VraSR, VCM	Hypothetical protein
SA2397		3.5	2.3		VCM	Similar to pyridoxal-phosphate dependent aminotransferase
SA2467	*hisH*	40.1	4.3		VCM	Amidotransferase HisH
SA2492	*vraD*	8.2	32.4	17.2	VCM	Similar to ABC transporter
SAS016		5.0	7.4	5.4	VCM	Hypothetical protein

*: Only the most strongly upregulated genes, the marker genes of the CAMP stimulon, are shown in this table. The complete list of genes induced at least 2-fold by several CAMPs is shown in additional file 1. In the case of operons, only the first gene or the most strongly induced gene is shown;
**: T, temporin L-NH_2; O, ovispirin-1-NH_2; D, dermaseptin K4-S4(1-16)-NH_2;
***: VCM, vancomycin inducible; VraSR, belongs to the VraSR regulon.

tion) is shown in additional file 1. The microarray data sets have been deposited in the GEO database (GPL7137 and GSE15800 for the complete microarray dataset) [32].

CAMPs induced the expression of the VraSR two-component system and consequently almost the whole VraSR regulon [33] was upregulated. In addition to the three antimicrobial peptides of animal origin, we also recently found that the human cathelicidin LL-37 induces the VraSR regulon (data not shown). Among the most strongly induced genes were *vraDE*, SA0205 and SAS016, which encode an ABC-type transporter similar to a putative bacitracin efflux pump [34,35], a lysostaphin-like cell-wall peptidase and a functionally-unknown peptide of 55 amino acids, respectively. The antimicrobial peptide treatment caused stress that induced general stress genes such as the *ctsR-clpC* operon, *groELS* and *dnaJK*. The synthesis of cell components involved in combating oxidative stress, catalase (SA1170), a putative thioredoxin (SA2324) and thioredoxin reductases (*trxB*), were also among the induced genes. Furthermore, a feature of the transcriptome was upregulation of several amino acid-biosynthesis operons (*dap*, *his*, *leu*, and *thr* operons).

Genes involved in anaerobic energy metabolism or encoding virulence factors were down-regulated in CAMP-treated cells

There were also down-regulated genes in the transcriptomes. Temporin L-NH_2, ovispirin-1-NH_2 and dermaseptin K4-S4(1-16)-NH_2 decreased the expression of 219, 194 and 134 genes, respectively. Most of the genes were down-regulated by more than one peptide (additional file 2). Notably, all three peptides had a strong inhibitory effect on the expression of genes involved in energy metabolism under anaerobic conditions [36]. The genes encoding enzymes for nitrate respiration (*nar* and *nas* operons) and fermentation (*pflP*, *pflA*, *ictE*, and *adh1*) were strongly repressed. Another striking phenomenon is that antimicrobial peptides caused down-regulation of several virulence factors and their regulators (*saeRS* and *agr*). Among the down-regulated virulence factor genes were *hld*, *ssaA*, *sbi*, *hlgA*, *ssl11* (*set15*), *clfA*, *clfB* and *spa*. The negative effect was particularly strong on the expression of *hld*, *ssl11*, *clfA* and *clfB* in cells treated with ovispirin-1-NH_2.

qRT-PCR measurements of the differentially expressed genes

We used qRT-PCR to verify some of the most interesting gene expression responses. The most strongly upregulated genes of the transcriptome,*vraD* (SA2492), SA0205 and SAS016 were subjected to qRT-PCR. The activity of the VraSR regulon was determined with three genes, *vraS* (SA1701), *prsA* (SA1659) and SA1477. The DNA microarray data suggested that an operon encoding a putative PstB-like phosphate uptake system (SA1217-SA1221) was induced by ovispirin-1-NH_2 and dermaseptin K4-S4(1-16)-NH_2 but not by temporin L-NH_2 (Table 1 and additional file 1). This induction pattern suggests that the peptide-induced expression from the promoter of the SA1217-SA1221 operon may depend on the mode of action of the antimicrobial peptides and we therefore were interested in confirming the peptide-specific induction pattern by qRT-PCR. We determined the expression level of SA1220. We also determined two further genes, *vraF* (SA0616) and *dltA* (SA0793), which were induced about 2-fold in the microarrays only by ovispirin-1-NH_2. The ortholog of *vraF* in *B. subtilis* is *bceA*, which encodes the ATPase component of an ABC transporter. Since *bceA* is strongly induced by bacitracin [34] and moderately by linear cationic antimicrobial peptides [25], and since it is also important for bacitracin resistance, we were interested in determining whether *vraF* is induced in the CAMP-treated cells of *S. aureus*. Furthermore, *vraDE*, *vraFG* and the *dlt* operon are all regulated by the Aps (or GraRS) sensory system [37]. The *dlt* operon encodes proteins which modify wall teichoic acids and lipoteichoic acids with D-alanine and thereby modulate the cell wall charge [9]. The density of negative charge in the wall affects the sensitivity of the bacteria to CAMPs.

The gene expression changes of the induced genes were clearly higher in the qRT-PCR measurements than in the DNA microarray, a previously described characteristic [38,39], but overall consistency was found in the results. The SAS016, *vraD*, SA0205, SA1477 and *prsA* genes exhibited the strongest gene induction responses; over 100-fold induction of SAS016 and *vraD* was observed with ovispirin-1-NH_2 (Figure 1). Most of the genes responded more strongly to ovispirin-1-NH_2 than temporin-L-NH_2. This was particularly clear in the induction of *vraD* and SA1220, but also *vraF*, *dltA*, *prsA*, SAS016 and SA1477 responded in this manner. SA0205 was the only gene which was expressed at higher levels in temporin-L-NH_2-treated cells as compared to ovispirin-1-NH_2-treated cells. The gene expression responses to dermaseptin K4-S4(1-16)-NH_2 were in most cases similar to those of ovispirin-1-NH_2.

The negative effect of CAMPs on the expression of *ssaA*, *sbi* and SA0423 was verified by qRT-PCR. Consistently with the microarray results, these genes were down-regulated in cells treated with the peptides (Figure 1). However, the decrease in expression was clearly lower in the qRT-PCR measurements.

The amide at the C-terminus of ovispirin-1 is crucial for the high level induction of vraDE

The three peptides used in the DNA microarray analysis were modified with C-terminal amide groups. We studied whether the amide affects gene induction by comparing

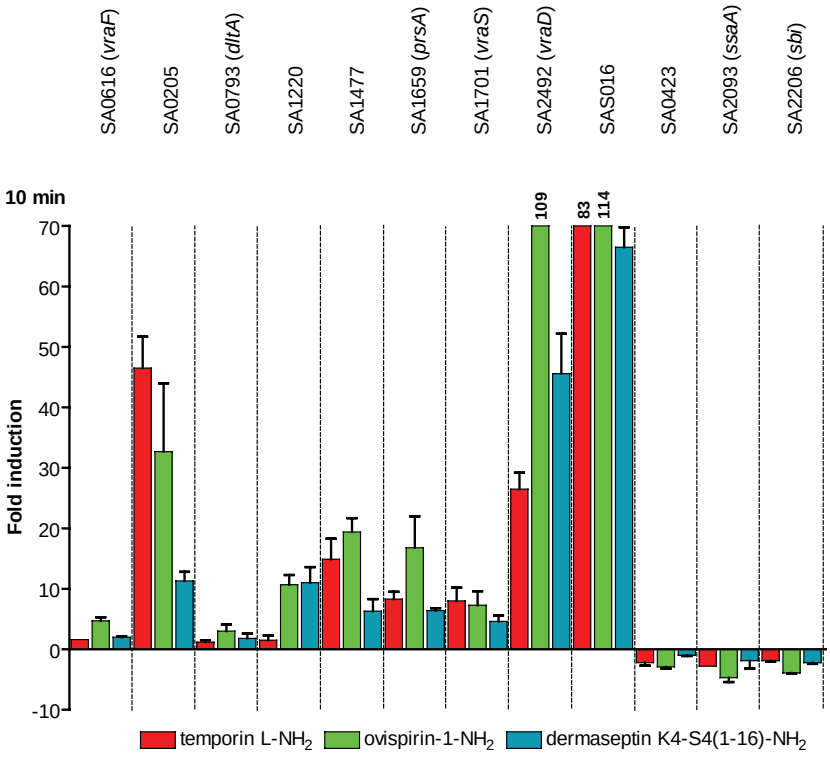

Figure 1

Induction or repression of genes in cells treated with cationic antimicrobial peptides as determined by qRT-PCR. The measurements of a set of differentially expressed genes were performed after 10 min treatments. The standard deviations of SAS016 are 19 (temporin L-NH_2) and 53 (ovispirin-1-NH_2). The standard deviation of *vraD* (ovispirin-1-NH_2) is 6.

the induction levels of four key genes in cells treated with amide-modified or non-modified ovispirin-1 or amide-modified temporin L. The determined genes were *groEL*, a general stress gene, *prsA*, the activity of which reflects the activity of the VraSR two-component system and the severity of the stress in the cell wall [33], and SA0205 and *vraD*, which were strongly induced by CAMPs. We also determined how other types of antimicrobial agents induce these genes. The antimicrobial agents were vancomycin and teicoplanin, which inhibit cell wall biosynthesis by interacting with the lipid II peptidoglycan precursor [40], bacitracin, an antimicrobial agent which interferes with the dephosphorylation of the peptidoglycan precursor [41], and daptomycin, a lipopeptide antibiotic with a specific mode of action on the cell membrane [42]. Furthermore, a pentaglycin peptide, A(D-Glu)K(D-Ala)GGGGGA(D-Glu)K(D-Ala), which mimics the peptide cross-link of *S. aureus* peptidoglycan, was tested to see whether a native cell wall peptide induces the expression of the four genes. This peptide was not antimicrobial at the concentration used, 20 μg/ml.

The results revealed that the C-terminal amide strongly influenced the induction of *vraD* by ovispirin-1. In the absence of the amide, *vraD* was only induced 3.7-fold (Table 2). In contrast, when ovispirin-1 was modified with the amide, *vraD* was induced 136-fold. The MIC values of both of the ovispirin-1 peptides for the *S. aureus* Newman strain were 20 μg/ml (Table 3), indicating that a difference in the stability of the peptides does not explain the large difference (about 35-fold) in the induction levels. The amide-modified peptide was also a better inducer of SA0205, *prsA* and *groEL*, but the difference was much smaller, only 2-4-fold. The induction pattern of the cell wall antibiotics vancomycin and teicoplanin was similar to that of ovispirin-1 without the C-terminal amide: the induction levels of SA0205 and *prsA* were clearly higher than those of *vraD* and *groEL*. The *vraD* expression was also strongly induced by bacitracin, almost 500-fold,

whereas the induction levels of SA0205 and *prsA* were much lower, 30-50-fold. Thus the induction pattern was similar to the amide-modified ovispirin-1. In contrast, temporin L-NH_2, despite the C-terminal amide and lower MIC than ovispirin-1-NH_2, was a poor inducer of *vraD* (Table 2, Figure 1) and resembled in this respect the non-modified ovispirin-1 and the cell wall antibiotics. The pentaglycine peptide did not induce any of the four genes.

VraDE is an ABC transporter system dedicated to resist bacitracin, whereas VraSR two-component system affects the resistance against a wider spectrum of antimicrobial agents

Our experiments showed that the VraDE ABC transporter and the VraSR two-component system are two CAMP-inducible systems which could be important for bacterial cells in resisting the harmful effects of CAMPs and possibly other antimicrobial agents. In order to study this, we constructed *vraDE* and *vraSR* null mutants and cultivated the mutants and the parental *S. aureus* Newman strain in Mueller-Hinton broth in the presence of various concentrations of the antimicrobials (two-fold serial dilutions) and determined their MIC values (Table 3). The CAMPs used in the susceptibility tests were, ovispirin-1-NH_2, ovispirin-1, temporin L-NH_2, two lantibiotics nisin and Pep5, human cathelicidin LL-37, and hBD3 defensin (the latter three are not shown in Table 3). In addition, we determined MICs for vancomycin, teicoplanin, bacitracin and daptomycin.

The mutations were verified by PCR (see methods), but also the expression of the *vraD*, SA0205, *prsA* and *groEL* genes in the *vraDE* and *vraSR* null mutants exposed to vancomycin was determined by qRT-PCR. The Δ*vraDE* mutant did not express *vraD* mRNA, consistent with the mutation (Table 4). The SA0205, *prsA* and *groEL* genes were expressed at about the same level as in the wild-type, 3-60-fold induction (Tables 4). In the Δ*vraSR* mutant, SA0205 and *prsA* were expressed at a low level (3-fold induction) consistent with the VraSR defect and suggesting that not only *prsA* but also SA0205 belong to the VraSR regulon. Their induction at a low level in the absence of VraSR may indicate that the expression is also controlled by another sensory system.

The Δ*vraDE* mutant was clearly more sensitive than the wild-type strains to bacitracin (about 10-fold difference in MIC), but no other differences were observed in the antimicrobial sensitivities. This strongly suggests that VraDE is a bacitracin-specific detoxification pump. More differences were observed with the Δ*vraSR* mutant. It exhibited increased sensitivity to teicoplanin (4-fold difference), bacitracin (4-fold difference) and ovispirin-1-NH_2 (2-fold difference). The Δ*vraSR* mutation did not affect the sensitivity to ovispirin-1, nisin, daptomycin, Pep5, LL-37 or hBD3. The same mutations in another *S. aureus* strain (RN4220) caused similar antimicrobial sensitivity differences, indicating that they are not strain specific (additional file 3).

In order to characterize further the phenotypes of the *vraDE* and *vraSR* null mutants, we subjected them to phenotype microarray (PM) analysis (Biolog). The analysis was performed with the PM11-20 sensitivity plates, which

Table 2: Induction of *vraD*, SA0205, *prsA* and *groEL* in *S. aureus* cells treated with peptides or cell wall-active antibiotics.

Antimicrobial agent and concentration**	Fold induction*			
	Gene			
	vraD	**SA0205**	***prsA***	***groEL***
Ovispirin-1 100 μg/ml	3.7 (0.5)	5.1 (1.0)	7.2 (1.6)	2.5 (0.5)
Ovispirin-1-NH_2 100 μg/ml	136.7 (8.7)	20.0 (0.7)	30.7 (1.7)	3.8 (0.4)
Temporin-L-NH_2 2 μg/ml	4.6 (1.3)	15.1 (3.6)	6.4 (1.8)	4.0 (0.1)
Bacitracin 100 μg/ml	467.5 (15.3)	51.4 (0.8)	27.9 (4.5)	3.6 (0.1)
Vancomycin 5 μg/ml	6.6 (0.6)	25.5 (1.8)	32.7 (1.7)	1.9 (0.0)
Teicoplanin 2.5 μg/ml	2.5 (0.3)	35.7 (6.9)	32.1 (4.1)	3.0 (0.3)
Pentaglysin 20 μg/ml	1.0 (0.0)	1.3 (0.1)	1.3 (0.0)	1.1 (0.0)

*: The ratio of the expression level of the gene in *S. aureus* Newman treated for 10 minutes with the antimicrobial agent or peptide to that in untreated cells as determined by qRT-PCR. The induction ratios are means from three experiments. The standard deviations are in parenthesis;
**: The concentrations of the antimicrobials and peptides used in the analysis are 5 × MIC (see Table 3) with the exception of temporin-L-NH_2, which was used at a subinhibitory concentration, and pentaglycine, which was not microbicidal at the 20 μg/ml concentration.

Table 3: Antimicrobial sensitivity of the Δ*vraDE* and Δ*vraSR* mutants and their parental strain *S. aureus* Newman.

Antimicrobial agent	MIC (μg ml^{-1})		
	Δ*vraSR*/*S. aureus* Newman	Δ*vraDE*/*S. aureus* Newman	*S. aureus* Newman
Ovispirin-1	20	20	20
Ovispirin-1-NH_2	10	20	20
Temporin L-NH_2	6	6	6
Nisin	6.25	6.25	6.25
Bacitracin	5	2.5	20
Daptomycin	2.5	2.5	2.5
Teicoplanin	0.125	0.5	0.5
Vancomycin	1	1	1

allow the testing of 960 phenotypes and the identification of increased or decreased sensitivities of the mutants as compared to the wild-type reference strain to a wide variety of non-peptide antimicrobial agents. Phenotype microarray analysis measures the reduction of tetrazolium dye (see experimental section).

The Δ*vraSR* mutant was more sensitive than the wild-type strain to a number of cell wall-active antibiotics including several cephalosporins (additional files 4 and 5). This result is consistent with a similar result of a previous study on a *vraSR* null mutant of *S. aureus* N315 [33]. The PM assay also suggested enhanced sensitivity to some other antimicrobial compounds than antibiotics. We verified the growth inhibitory effects of four of these antimicrobials, cefotaxime, chlorpromazine, EGTA and sodium tungstate, by cultivating the Δ*vraSR* mutant and the wild-type *S. aureus* Newman strain in BHI medium on microtiter plates containing 0.31 μg/ml, 0.8 mM, 31.25 μg/ml and 25 mM concentrations of these antimicrobials, respectively, and measuring the optical densities of the cultures. The Δ*vraSR* mutant was unable to grow in the presence of these antimicrobials, while the wild-type strain grew significantly (Figure 2). In addition to these major differences of sensitivity, several minor differences were also observed, but we did not verify them in microtiter plate cultures by optical density measurements. The *vraSR* null mutant was resistant to macrolide antibiotics due to the *ery* gene used in the mutant construction. The PM analysis of the Δ*vraDE* mutant did not reveal any significant sensitivity differences (not shown), suggesting again a dedicated role for VraDE in bacitracin resistance.

Table 4: Induction of *vraD*, SA0205, *prsA* and *groEL* in *S. aureus* Δ*vraSR* and Δ*vraDE* mutants treated with vancomycin.

Mutant	Fold induction*			
	Gene			
	vraD	SA0205	*prsA*	*groEL*
Δ*vraSR*	7.9 (0.2)	2.5 (0.2)	3.2 (0.1)	2.1 (0.1)
Δ*vraDE*	not expressed	60.4 (2.1)	36.0 (0.7)	3.1 (0.4)
wild type	6.6 (0.6)	25.5 (1.8)	32.7 (1.7)	1.9 (0.0)

*: The ratio of the expression level of the gene in the mutant or the wild-type strain treated for 10 minutes with 5 μg/ml of vancomycin to that in untreated cells as determined by qRT-PCR. The induction ratios are means from three experiments. Standard deviations are in parenthesis.

Discussion

A prominent feature of the CAMP transcriptomes of *S. aureus* Newman was the induction of the VraSR regulon. The regulon consists of several genes involved in cell wall-associated functions such as protein quality control, protein folding and modulation of cell wall biosynthesis [33]. The induction pattern of the VraSR-regulated genes was very similar to that observed with vancomycin-treated cells in another transcription profiling study with another *S. aureus* strain, N315 [33]. In addition to the effects on membranes, several antimicrobial peptides such as nisin, mersacidin and bacitracin inhibit cell wall biosynthesis [41,43-45]. Whether the VraSR-inducing linear CAMPs used in this study inhibit cell wall biosynthesis and cause a cell wall defect cannot be judged from our data, but this is the most likely explanation for the similar induction patterns of the VraSR-regulated genes. A transcription profiling study with a lipopeptide antibiotic daptomycin also showed up-regulation of the the VraSR cell wall regulon [46], which is probably caused by an inhibitory effect on the cell wall biosynthesis [47], although the results furthermore suggested that some of the up-regulated genes in the daptomycin transcriptome were induced by its membrane depolarization effect [46]. A similar dual effect has also been shown with human β-defensin 3 [48].

In *B. subtilis*, the *yxdLM* operon, which encodes an ABC transporter, is strongly induced by human cathelicidin LL-37 in a manner dependent on the YxdJK two-component system, but not at all by porcine protegrin PG-1 and a model antimicrobial peptide poly-L-lysine [25]. This indicates that antimicrobial peptides can be very specific in activating stress sensors and the activation mechanism

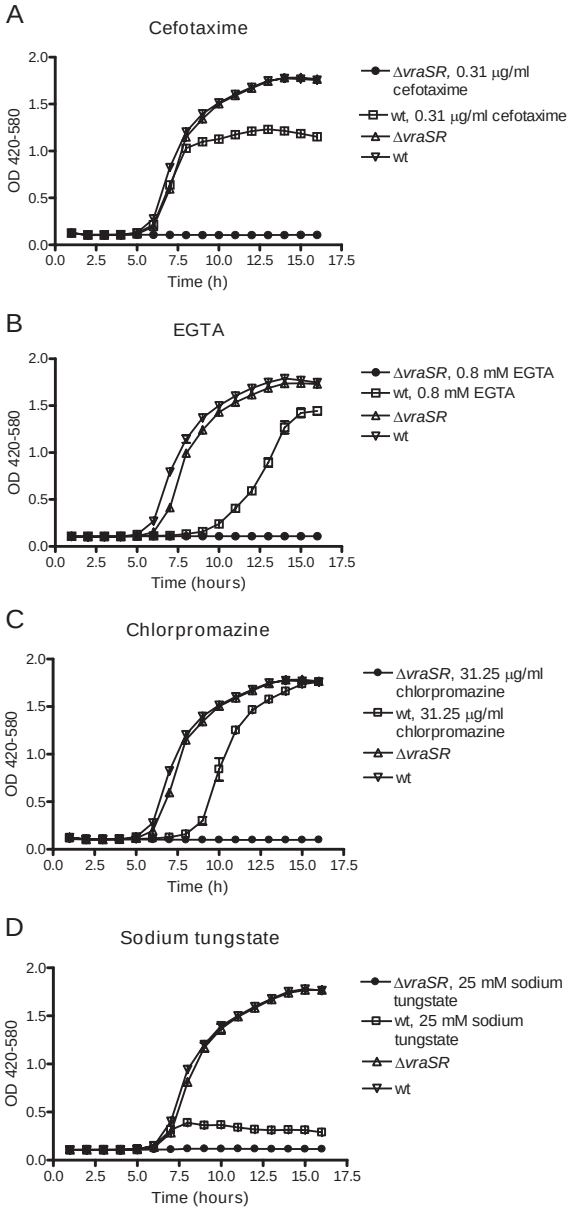

Figure 2

Increased sensitivity of the Δ*vraRS* mutant to cefotaxime, EGTA, chlorpromazine and sodium tungstate. The Δ*vraRS* mutant and the wild-type *S. aureus* Newman strain were grown either in the presence or absence of the antimicrobial agents as indicated in 150 μl of BHI medium in wells of microtiter plates in a Bioscreen for 16 hours. The optical densities of the cultures were measured every 15 minutes. The data points show the densities measured at one-hour intervals.

may even be dependent on the mode of action, charge or structural properties of the peptides. In this study we found that the *vraDE* ones were the most strongly induced genes in cells treated with ovispirin-1-NH_2 or dermaseptin K4-S4(1-16)-NH_2. We could demonstrate that the high level of induction of *vraDE* with ovispirin-1-NH_2 was dependent on the amide group at the C-terminus. The antimicrobial effects of these two variants of ovispirin-1 on wild-type *S. aureus* Newman strain were not different. The expression regulation of *vraDE* is under the control of the Aps (GraRS) sensor system, which also controls the expression of *vraFG*, encoding a putative peptide efflux pump, *dlt* operon, encoding components responsible for the D-alanylation of teichoic acids and modulation of the net negative charge of the cell wall, and *mprF*, encoding the MprF enzyme which catalyzes the lysinylation of phosphatidylglycerol and modulates the charge of the outer surface of the cell membrane [37]. It has been shown that a nine-aminoacid loop of the ApsS sensor exposed on the outer surface of the membrane interacts with CAMPs when activating the sensor [37]. Our results suggest that in linear peptides the C-terminal amide group is an important element for the activation of the ApsS sensor. In the study by Li and collaborators [37] the peptides which naturally contain an amide group at the C-terminus, indolicidin and melittin, were significantly better inducers of the *dltB* gene than those that do not have the amide such as magainin II and nisin. The *vraDE* genes were also the most strongly induced genes in the stress response to C-terminally amide-modified human cathelicidin LL-37 (our unpublished results). However, the low level of induction of *vraDE* with temporin L-NH_2 in this study suggests that the C-terminal amide is not the only element that is recognized by the ApsS sensor and needed for the induction. Alternatively, the level of the induction of the Aps regulon might rather depend on the absence of the carboxyl group than on the presence of the amide. Mersacidin, which is a lantibiotic, but unlike nisin, does not have a free C-terminal carboxyl group due to an intramolecular thioether-ethyleneamide bridge, is a very strong inducer of *vraDE* [49] and might fit this model.

VraDE is a transporter system that is dedicated to resist bacitracin, as evidenced in this study by the testing the sensitivity of the *vraDE* null mutant(s) to 11 antimicrobial peptides or antibiotics and numerous other antimicrobial agents (PM analysis). The Δ*vraDE* mutant(s) was more susceptible to bacitracin but not to any other antimicrobial tested. Consistently, VraDE is similar to the BceAB bacitracin transport system of *B. subtilis* [34,35] and probably comprise together with VraFG an efflux pump system for combating bacitracin [37]. A recent study suggested that in *B. subtilis* bacitracin sensing and the expression of the *bceAB* genes are dependent on active bacitracin transport via BceAB and that the large periplasmic loop of the BceA permease component of the transporter may be involved in mediating the signal to the BceRS two-component system [50]. We found that bacitracin is a strong inducer of *vraDE* expression. Whether the sensing mechanism of Aps is dependent on the transport function of VraDE or VraFG, whether Aps senses linear CAMPs and bacitracin in a similar manner and whether the amide group of the side chain of glutamine in the cyclic bacitracin peptide [51] have a similar essential role in the activation, should be addressed in future studies. The C-terminal amide being in a sequence contex that is appropriate for the interaction with the sensory loop of ApsS most probably explains the differences in the induction patterns of the *vraDE*, *vraF* and *dltA* genes observed in this study. However, we can not rule out the possibility that the carpet-mode of action mechanism influences the induction, since the amide-modified carpet peptides ovispirin-1-NH_2 and dermaseptin K4-S4(1-16)-NH_2 were clearly stronger inducers of these genes than the amide-modified toroidal pore-forming peptide temporin L-NH_2.

In addition to *vraDE*, SA0205 was strongly induced by CAMPs, but in this case the strongest inducer was temporin L-NH_2. Our results indicated that SA0205, which encodes a putative cell membrane-associated peptidase, belongs to the VraSR regulon. The over 100-fold induced (qRT-PCR) SAS016 encodes a small protein with unknown function and mechanism of regulation. It was also strongly induced by vancomycin [52], suggesting that it responds to a cell wall defect. Furthermore, it was detected at both transcript and protein levels in GISA strains [53]. *S. aureus* cells try to adapt to the stress and harmful effects of CAMPs by inducing or repressing several gene systems. These responses include the induction of the *ctsR*, *dnaJ*, *dnaK*, *hrcA*, *groEL* and *groES* general stress genes and genes resisting oxidative stress (*katA*, *trxB* and SA2324). Some of these targets showed similar inductions in *S. aureus* during phagocytosis by neutrophils or when surviving within epithelial and phagocytic cells [54,55]. Notably, several amino acid biosynthesis operons and genes encoding enzymes of the citric acid cycle were also induced and genes involved in anaerobic metabolism were repressed, indicating that the cells were metabolically active and respiring aerobically. The down-regulation of virulence gene expression is partly caused by the repression of *saeRS*, but also the decreased expression of the *agr* operon affects their low expression.

S. aureus is a human pathogen and it can be argued that the CAMPs of animal origin may cause a different kind of transcriptional response than CAMPs of the natural host. A transcriptional analysis of responses of *S. aureus* (SG511) to human β-defensin 3 (hBD3) showed that *vraDE*, *vraSR* and SA0205 were also induced by this human CAMP [48], as was the case with the animal

CAMPs in this study. The most notable difference in the transcriptomes is the very strong induction of the SA0192 gene, which encodes a protein similar to ABC transporter ATP-binding proteins, in SG511 cells exposed to hBD3. Since this gene is absent in the genome of the *S. aureus* Newman strain, it is not among the induced genes of this study. Sass and collaborators recently showed that a knockout mutation of *vraE* increases the susceptibility of *S. aureus* SG511 also to other antibiotics than bacitracin, including antimicrobial peptides hBD3, LL-37, Pep5 and nisin [48]. The defective GraRS/Aps sensory system of the strain SG511 [56] may explain the inconsistency with the result of this study.

The Δ*vraSR* mutant exhibited enhanced sensitivity to several antimicrobial agents as compared to the wild-type strains. They were more sensitive to the amide-modified ovispirin-1 but not to the non-modified one. Ovispirin-1-NH_2 was a better activator of Aps and VraSR than ovispirin-1 and this may explain the higher resistance of the wild-type strain to the amide-modified peptide. The Δ*vraSR* mutation also decreased the MIC values of bacitracin and teicoplanin. This effect is similar to what has been observed with a Δ*vraSR* mutant of *S. aureus* N315 [33]. Our phenotype microarray analysis revealed increased sensitivity of the *S. aureus* Newman Δ*vraSR* mutant to a wide range of β-lactam antibiotics. Furthermore, we showed that Δ*vraSR* increased susceptibility to other types of antimicrobial agents such as EGTA and phenothiazines. Since EGTA is a metal-chelator, it probably harmfully affects bacterial cells and particularly severely Δ*vraSR* mutant cell by binding to divalent metal cations in the cell wall and consequently causing metal sequestration. The phenothiazines chlorpromazine and thioridazine, which are or have been used in psychiatric therapy, are potential lead compounds for the development of new cell wall-active antibiotics. It has been shown that the dosage of chlorpromazine used in psychiatry results in intracellular concentrations of this drug that are antimicrobial [57].

Conclusion

The current rapid increase in antibiotic resistance among human pathogens coincides dangerously with a lack of novel antibiotic discovery. A promising alternative is a class of short peptides known as antimicrobial peptides, or host defense peptides, which are found among all classes of life but have yet to be widely exploited for pharmaceutical purposes. In order to identify the inducible resistance mechanism of *Staphylococcus aureus* against antimicrobial peptides and other antimicrobial agents, the bacterium was exposed to antimicrobial peptides and gene expression changes were analyzed. The analysis revealed similar gene induction patterns with cell wall-active antibiotics but also some distinctly different ones. The induction of the VraSR regulon suggests that antimicrobial peptides have an inhibitory effect on the cell wall biosynthesis. This must be verified biochemically in future studies. Parts of the signalling pathways are not specifically dedicated to CAMP defense but are regulated following the lifestyle of pathogenic bacteria which are potentially exposed to a wide diversity of environmental changes.

This study does not provide a definitive answer as to whether the mode of action of CAMPs on lipid bylayers is responsible for certain induction patterns, but this is a potential alternative. For instance in the case of the induction of the SA1217-SA1221 operon, the carpet-forming peptides ovispirin-1-NH_2 or dermaseptin K4-S4(1-16)-NH_2 were strong inducers whereas the pore-forming peptide temporin L-NH_2 was unable to induce this operon. Most importantly, it was found that modifying the carboxy-terminus of a linear peptide with an amide group can modulate drastically its properties as an inducer of resistance mechanisms. This result may allow the rational design of antimicrobial agents that lack the properties to induce resistance mechanisms. Inactivating the VraSR cell wall stress management system increased susceptibility of the bacterium to a wide range of cell wall-active antibiotics but also susceptibility to several non-antibiotic antimicrobials was increased. Particularly interesting in the latter group are the established antipsychotic drugs, chlorpromazine and thioridazine, which are potential lead molecules for developing novel antibiotics.

Methods

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used are listed in Table 5. *E. coli* cells were grown in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg ml^{-1}) and *S. aureus* cells in BHI (Brain Heart Infusion) and TSB (Tryptone Soya Broth) media supplemented with chloramphenicol (10 μg ml^{-1}) and/or erythromycin (2.5 μg ml^{-1}) when needed. To determine the subinhibitory concentrations of temporin L-NH_2 (FVQWFSKFLGRIL-NH_2) ovispirin-1-NH_2(KNLRRIIRKIIHIIKKYG-NH_2) and dermaseptin K4-S4(1-16)-NH_2 (ALWKTLLKKVLKAAAK-NH_2), 150 μl of BHI medium was inoculated with approximately 10^5 *S. aureus* Newman cells in honeycomb 2 plate wells, peptides were added in two-fold dilution series, and the cultures were incubated with continuous and moderate shaking at 37°C and culture densities were measured in a Bioscreen C Microbiology reader (Growth Curves, Helsinki, Finland). The peptide treatments for the gene expression analyses were carried out in the exponential growth phase (~OD_{560} = 0.6); CAMPs were added at the final (sublethal) concentrations of 3 μM (temporin L-NH_2), 4 μM (ovispirin-1-NH_2) and 3 μM (dermaseptin K4-S4(1-16)-NH_2) if not otherwise indicated. The addition of the peptides at these sublethal concentrations slowed down the growth slightly. The peptides were pur-

chased from EZbiolab (Carmel, IN, USA) and their purity was 95%. The Minimal Inhibitory Concentration (MIC) determinations were performed by cultivating *S. aureus* Newman or RN4220 cells and their *vraSR* and/or *vraDE* null mutation derivatives in 100% (temporin L-NH_2, ovispirin-1-NH_2, ovispirin-1, nisin, vancomycin, bacitracin, teicoplanin, daptomycin and pentaglysine), 50% (LL-37, nisin and Pep5) or 25% (hBD3) Mueller-Hinton broth for 16-24 hours. Typically, an overnight grown culture was diluted 1/10000 and 1 ml of broth in a 15 ml sterile Falcon tube was inoculated with 10 μl of the diluted culture and cultivated at 37°C with shaking (220 rpm min^{-1}). Each MIC determination was performed twice with a two-fold dilution series of the antimicrobial agents.

Isolation of total cellular RNA

The samples for RNA isolations were taken 10 minutes after the addition of the antimicrobial peptides or other antimicrobial agents at the cell density of OD_{600} = 0.6 or Klett 50. The cells from 1.8-2 ml of the cultures (BHI medium) were harvested by centrifugation. The supernatants were discarded and the bacterial pellets were frozen in liquid nitrogen if the RNA isolation was not conducted immediately. The control samples without peptides were treated in a similar manner. The cell pellets were resuspended in 200 μl TE-buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.0) containing lysostaphin (125 μg ml^{-1}) and incubated at 37°C for 10 minutes. RNA was extracted by using Roche's High Pure RNA Isolation Kit according to the manufacturer's instructions.

Transcriptional analysis by oligo DNA microarray

The effects of the three different antimicrobial peptides on gene expression of *S. aureus* Newman were studied by using whole genome oligo-DNA microarrays. Bacteria were grown in BHI medium to the early exponential phase (OD_{600} = 0.6) and antimicrobial peptides were added at the sublethal concentrations described above. Samples were taken for RNA isolations after treating the cultures with the peptides for 10 minutes. Control cultures without peptide additions were treated similarly and in parallel. Genes with at least 2-fold induction/reduction of expression in the peptide-treated cells as compared to the control cells (non-treated) were accepted as differentially expressed genes.

The microarray was manufactured by *in situ* synthesis of 10'807, 60-mer oligonucleotide probes (Agilent, Palo Alto, CA, USA), selected as previously described [38]. It covers >98% of all ORFs annotated in strains N315 and Mu50 [58], MW2 [59] COL [60], NCTC8325 (the sequence, locus NC_007795, can be found at the National Center for Biotechnology Information [61]), USA300 [62], MRSA252 and MSSA476 [63] including their respective plasmids. Extensive experimental validation of this array has been described previously, using CGH (comparative genomic hybridization), mapping of deletion, specific PCR and quantitative RT-PCR [38,64].

Total RNA was further purified with Qiagen RNeasy mini kit and treated with DNAse following the manufacturer's recommendations. The absence of remaining DNA traces was evaluated by quantitative PCR (SDS 7700; Applied Biosystems, Framing-ham, MA) with assays specific for 16s rRNA [53,65]. Samples of 8 μg total *S. aureus* RNA were labelled by Cy-3 or Cy-5 dCTP using the SuperScript II (Invitrogen, Basel, Switzerland) following the manufacturer's instructions. Labelled products were then purified with QiaQuick columns (Qiagen). A mixture of control (without peptide) and test conditions (with peptide) was then diluted in 250 μl Agilent hybridization buffer, and

Table 5: Bacterial strains and plasmids.

Strain/Plasmid	Description	Reference/Origin
E. coli		
5-alpha	cloning strain	New England Biolabs
S. aureus		
Newman	ATCC25904, methicillin susceptible	[72]
RN4220	NCTC 8325-4-r, restriction negative strain	[73]
COL	methicillin resistant (MRSA)	[60]
RH7657	Newman Δ*vraSR*::ery	this study
RH7661	Newman Δ*vraDE*::ery	this study
RH7788	RN4220 Δ*vraDE*::ery	this study
RH7790	RN4220 Δ*vraSR*::ery	this study
Plasmids		
pGEM-3zf(+)	cloning vector, amp^r (*E. coli*)	Promega
pKOR1	gene replacement vector, amp^r (*E. coli*), chl^r (*S. aureus*)	[69]
pKTH3762	pGEM-3zf(+) containing the *vraSR* deletion cassette, amp^r (*E. coli*)	this study
pKTH3763	pKOR1 containing the *vraSR* deletion cassette, amp^r (*E. coli*), chl^r ery^r (*S. aureus*)	this study
pKTH3764	pGEM-3zf(+) containing the *vraDE* deletion cassette, amp^r (*E. coli*)	this study
pKTH3765	pKOR1 containing the *vraDE* deletion cassette, amp^r (*E. coli*), chl^r ery^r (*S. aureus*)	this study

hybridized at a temperature of 60 °C for 17 hours in a dedicated hybridization oven (Robbins Scientific, Sunnyvale, CA, USA). The slides were washed with Agilent proprietary buffers, dried under a nitrogen flow, and scanned (Agilent, Palo Alto, CA, USA) using 100% PMT (photomultiplier tube) power for both wavelengths.

Fluorescence intensities were extracted using the Feature extraction™ software (Agilent, version 8). Local background-subtracted signals were corrected for unequal dye incorporation or unequal load of labelled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally weighted linear regression) method. Data consisting of three independent biological experiments were analyzed using GeneSpring 7.3 (Agilent) after per gene and per chip normalization. The statistical significance of differentially expressed genes was identified by variance analysis (ANOVA) [53,66], performed using GeneSpring, including the Benjamini and Hochberg false discovery rate correction (5%). Genes showing significant changes ($P < 0.05$) and at least a 2-fold induction/reduction of expression, in the peptide-treated cells as compared to the control cells (non-treated) were accepted as differentially expressed.

Quantitative real-time RT-PCR

cDNA was synthesized with 1 μg of total cellular RNA by using a High Capacity cDNA Archive Kit (Applied Biosystems) with an additional step of DNase I (Roche) treatment [67] The quantitative real-time PCR was performed as previously described [25] by using specific primer pairs and a SYBR Green PCR system kit (Applied Biosystems). The primers (additional file 6) were purchased from TAG Copenhagen. The amplification reactions and detection of PCR products were performed with a 7500 real time PCR system (Applied Biosystems). The cDNA values were normalized with the value of *gyrA*.

Construction of the vraDE and vraSR null mutants

In order to construct null mutants of the *vraDE* and *vraSR* operons, about 1 kb DNA fragments of both the upstream and downstream regions of the target operons were PCR amplified. The template DNA was *S. aureus* chromosome either from the strain Newman (*vraSR*) or COL (*vraDE*). The primers used in the PCR reactions are shown in additional file 6. The upstream fragments digested with *Pst*I and *Sal*I and the downstream fragments digested with *Bam*HI and *Eco*RI/*Sac*I were ligated with a PCR-amplified DNA fragment containing the erythromycin resistance gene of pMUTIN4 [68]. The resulting *vraDE* and *vraSR* inactivation cassettes were inserted into the pGEM3Zf(+) plasmid vector between the *Pst*I and *Eco*RI/*Sac*I sites, followed by transformation of competent *E. coli* 5-alpha (NEB) cells with the ligated DNA. The inactivation cassettes were then PCR amplified by using the pGEM3Zf(+) constructs as PCR templates and specific oligonucleotides, containing the sequences of attB1 and attB2 sites, as PCR primers. The amplified fragments were inserted into the pKOR1 gene replacement plasmid in a recombination reaction with Clonase (Invitrogen) as previously described [69]. The resulting pKOR1-inactivation cassette constructs were used to transform electrocompetent *S. aureus* RN4220 cells by electroporation [70] and transformants were selected by cultivating them on BHI agar plates supplemented with chloramphenicol (10 μg ml^{-1}) and erythromycin (2.5 μg ml^{-1}) at 30 °C. The gene replacement plasmids were isolated from the RN4220 transformants and subsequently electroporated into *S. aureus* Newman cells. The mutant construction was performed as described [69]. Briefly, *S. aureus* Newman or RN4220 cells harbouring the gene replacement plasmids were cultivated in TSB medium overnight at 43 °C, a non-permissive temperature for the replication of pKOR1 and its derivative plasmids, to force the plasmids to integrate into the chromosome by a Campbell-type recombination at a flanking region of the target gene. The resulting merodiploid cells were cultivated on TSA plates containing 100 ng ml^{-1} anhydrotetracyclin but not chloramphenicol to induce pKOR1-encoded *secY* antisense transcripts and counter-select cells which via homologous recombination between the gene duplicates had lost the pKOR1. The presence of erythromycin (2.5 μg ml^{-1}) on the plates allowed the selection of the mutants. The mutants were verified by PCR with appropriate primers (additional file 6).

Phenotype microarray (PM) analysis of the vraDE and vraSR null mutants

The PM analysis was carried out by using the service of Biolog (Hayward, CA, USA). We wanted to identify antimicrobial agents and stress conditions which are more harmful to the mutant cells than the wild-type *S. aureus* Newman cells. Therefore, the analysis was performed only with sensitivity panels 11-20, which allowed the testing of 960 stress conditions and 240 different antimicrobial agents on ten 96-well plates. The method has been described more thoroughly in a previous study [71]. Briefly, the growth of bacteria in a well of the PM panels can be quantitated since growing, metabolically-active bacteria reduce the tetrazolium indicator dye and blue color is formed. Bacteria were grown in BHI medium for 24 hours in an OmniLog instrument. The color changes of the redox dye in the cultures were compared (mutant *versus* wild type). The formation of the reduced tetrazolium was recorded either as a green tracing (mutant) or a red tracing (wild-type strain). The OmniLog PM bioinformatics software was used to overlay the color-coded images of the kinetics of the tetrazolium reduction and compare and quantify them. Each strain was analyzed twice and con-

sensus images were obtained showing the differences in antimicrobial sensitivity that were detected in both PM runs.

Authors' contributions

Experimental planning: VPK. Performed DNA microarrays and data analysis: PF JS MP VPK. Performed qRT-PCR measurements: MP HLH. Constructed mutants: MP VPK. Performed antimicrobial sensitivity measurements: MP VPK VS HGS. Wrote the paper: MP PF VPK.

Additional material

Additional file 1

***Genes and operons induced by several cationic antimicrobial peptides**. The table shows the genes induced at least 2-fold by several cationic antimicrobial peptides.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-429-S1.doc]

Additional file 2

***Genes and operons down-regulated by several cationic antimicrobial peptides**. The table shows the genes down-regulated by several cationic antimicrobial peptides.*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-429-S2.doc]

Additional file 3

***Antimicrobial sensitivity of* S. aureus *RN4220 and its Δ*vraDE *and Δ*vraSR *mutant derivatives**. The table shows MIC values of antimicrobial peptides and other antimicrobial agents against* S. aureus *RN4220 and its Δ*vraDE *and Δ*vraSR *mutant derivatives.*
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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-429-S3.doc]

Additional file 4

***Antimicrobial sensitivities of RH7657 (vraSR::ery) and RH7603 (S. aureus Newman) were compared by using phenotype microarrays**. Scatter plots of parameter values from two replicates of the PM analysis are shown in the two uppermost panels. The three other panels show overlaid color-coded images of tetrazolium reduction kinetics (mutant versus wild type) over all wells in the two runs of the analysis and their consensus.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-429-S4.doc]

Additional file 5

***Decreased reduction of tetrazolium in* vraSR *null mutant treated with antimicrobial agents as compared to the wild-type* S. aureus *Newman strain**. The gene list was obtained from the consensus of the two independent phenotype microarray analyses shown in additional file 4.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-429-S5.doc]

Additional file 6

***Specific primer pairs for qRT-PCR and PCR**. The table shows the primers used in this study.*
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